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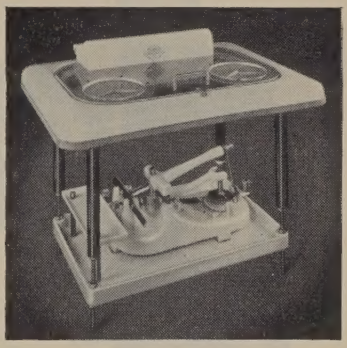

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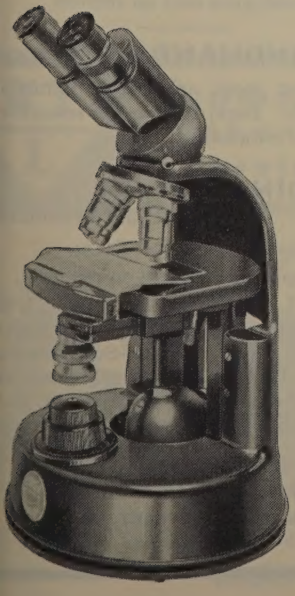

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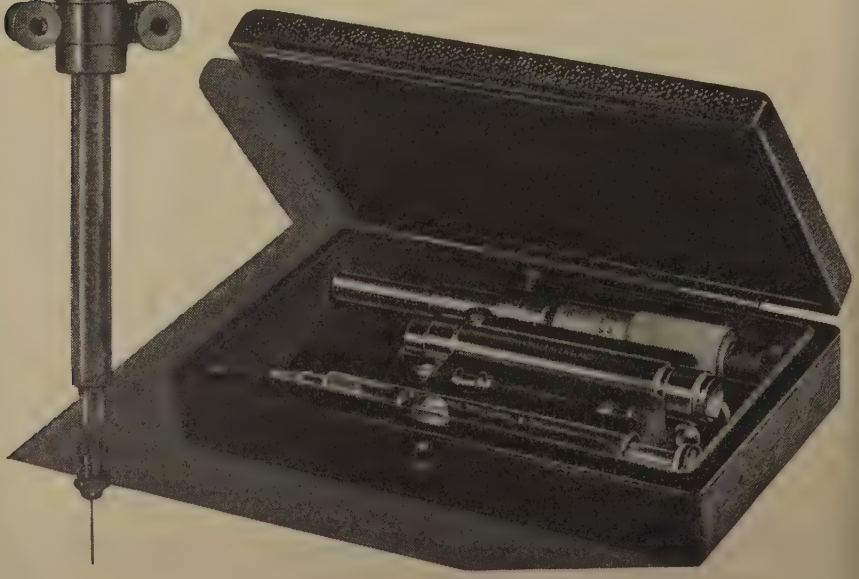

Principles and Technique of Fluorescence Microscopy

By M. R. YOUNG

(*From the National Institute for Medical Research, Mill Hill, London, N.W. 7*)

With four plates (figs. 3 to 6)

Contents

Summary

Interest in fluorescence microscopy has greatly increased in recent years. Technical considerations have to some extent prevented even wider application of the various fluorescence techniques now available for microscopical study of biological specimens. This paper outlines the basic requirements for optimal image quality, for the benefit of biologists and others who may not be conversant with the optical principles involved. The central problem of illumination is reviewed in some detail, and an assessment given of the two methods in current use, namely the bright-field and dark-field systems. Ratios of fluorescent to activating light received by the objective aperture, given by the two systems, have been compared, and measurements have been made of their relative light-concentrating power.

Available light sources and their suitability for the excitation of fluorescence are discussed, with the problems of selecting appropriate light filters for use with the alternative systems of illumination.

It is concluded that the dark-field system has decided advantages in practice and in theory for the following reasons:

(1) The dark-field condenser serves as an efficient primary filter, contributing to a black background and hence good contrast.

(2) The equivalent focal length is less than that of the bright-field condenser and it concentrates energy in a smaller area; this compensates in part for the loss of energy inevitably caused by the central stop.

(3) It permits the use of wide-band primary filters of maximum transmission because contrast in the fluorescent image is affected only by a weak superimposed dark-field image produced in the object-plane by scattered residual activating light passed by the primary filter. With blue-light activation the visible dark-field image is effectively eliminated by means of a weak blue-absorbing secondary filter.

(4) The loss of contrast due to veiling glare is minimized.

A rational layout for fluorescence microscopy and methods for accurate alignment of the microscope in the vertical and horizontal positions are described. Factors influencing the choice of suitable objectives and eyepieces and some details of methods for mounting specimens are given.

[Quarterly Journal of Microscopical Science, Vol. 102, part 4, pp. 419-449, 1961.]

Introduction

THE possibility of studying the distribution and morphology of autofluorescent structures in biological specimens was recognized by Köhler (1904 *a*, *b*) during early experiments in ultra-violet microscopy. Light from the cadmium spark at a wavelength of 275 mμ was used. Many objects exhibited fluorescence when illuminated in this way, or by an intense emission in the region of 280 mμ from the magnesium spark. Köhler further envisaged the possibility of treating microscopical objects to make them self-luminous, so anticipating the use of fluorescent dyes which were later introduced by Prowazek (1914).

Ellinger (1940) records how Köhler and Siedentopf in some pioneer work in 1908 tried out a dark-field condenser for concentrating ultra-violet light on to the specimens. The sub-stage illuminating components, specimen slide and coverglass were made of quartz; observation was with normal glass objectives and oculars. A fluorescence microscope incorporating these features but with a carbon arc as the light source was devised soon afterwards by Heimstadt (1911); but in spite of this early recognition of the value of dark-field illumination most investigators were satisfied with bright-field condensers for fluorescence work.

However, in 1937 Barnard and Welch used the Beck–Barnard ultra-violet microscope and a special dark-field condenser designed by Smiles (1933) for investigation of some fluorescent components in bacterial cells, and were responsible for renewed interest in the dark-field system. They showed subsequently that adequate low-power fluorescence microscopy could also be carried out with a bright-field condenser fitted with a central stop to prevent direct light entering the objective.

Today there is increasing interest in fluorescence microscopy, encouraged by introduction of the labelled-antibody technique (Coons and Kaplan, 1950) and by the application of fluorescent dyes as tracers and in histochemical techniques. Utilizing the special affinity of aminoacridine compounds for nucleic acids, Armstrong (1956) developed a sensitive fluorescence technique in which acridine orange is used for the identification of DNA and RNA in mammalian cells; this has been applied profitably to the study of cytochemical aspects of virus cytopathology (Anderson, Armstrong, and Niven, 1959). For routine detection of mycobacteria in smears or tissue sections, fluorescence microscopy after auramine staining has replaced the standard Ziehl–Neelsen method in some laboratories; and fluorescent dyes are now similarly employed in diagnostic exfoliative cytology (Friedmann, 1950; Bertalanffy, Masin, and Masin, 1956, 1958).

Many variations on the basic optical equipment for fluorescence microscopy are described in the literature, but in every case the apparatus falls into one of two categories depending upon the condensing system employed for illumination of the specimen. For the studies with acridine compounds, referred to above, the apparatus used was one developed for general fluorescence work at

he National Institute for Medical Research; this incorporates a dark-field ondenser of the cardioid type. Coons and his associates (1955) have evidently avoured a very similar arrangement for their more recently published work vith the fluorescent antibody technique. On the other hand, Ellinger (1940) onsidered the dark-field system to be obsolete on the grounds that it naturally imited the intensity of the exciting radiations; essentially the same view was xpressed in a recent and authoritative review by Richards (1955), and has een accepted by many microscopists. A primary object of the present paper, herefore, is to focus attention upon definite advantages of the dark-field ystem, deduced both from a consideration of the optical principles involved nd from practical experience with many of the current uses of fluorescence nicroscopy in biological research.

Methods of Illumination

Fluorescence occurs when a substance absorbs light of specific wavelengths nd simultaneously re-emits part of this energy at longer wavelengths, usually n the visible region of the spectrum. In order to observe fluorescence through he microscope it is necessary to illuminate the object with light of high in- ensity and of specific wavelengths, generally in the region between 300 mμ nd 500 mμ.

A fundamental difference between the ordinary light microscope and one dapted for observing fluorescence lies in the mode of formation of the visible nage. Normally, the image is formed by the modification of light passing hrough the specimen, and to obtain well-resolved images with good contrast he aperture of the condenser should not exceed that of the objective. A fluor- scent image, on the other hand, is due to visible light emanating from the pecimen itself, and the illuminating beams which excite fluorescence do not ontribute directly to the formation of the image. In view of the low intensity f most forms of fluorescence and inevitable light losses of up to 90% in the nicroscope, it becomes essential to employ the most efficient possible light ource and optical system for illumination of the specimen. In the fluorescence nicroscope therefore the illuminating aperture must be as large as working onditions will permit to ensure that the maximum amount of light will reach he specimen.

The quality of the fluorescent image, and the precise requirements for ptimal observing conditions, depend ultimately on physical properties of the pecimen itself. The most satisfactory results can be anticipated only when uch properties have been taken into account in the layout of the optical ystem. When fluorescent materials are irradiated with light, maximal excita- on occurs in the regions where there is a high level of energy absorption. The ost suitable exciting wavelengths for fluorescence microscopy will be deter- ined therefore mainly by the absorption characteristics of the specimen nder investigation, e.g. porphyrins naturally occurring in tissues having pecific absorption maxima at 400 mμ and 550 mμ; but much of the recent ork on biological specimens has involved the use of fluorescent dyes or

labelling reagents and in these circumstances it is the absorption properties o the reagents (or, more precisely, of the tissue-reagent complex) which deter mine the appropriate conditions for microscopical observation. Absorptio curves of some of the fluorescent dyes in general use are shown in fig. 1. Apa

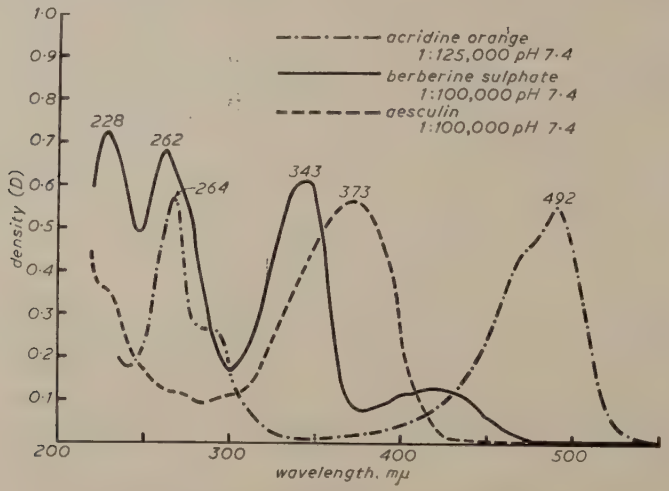

FIG. 1. Spectral absorption curves of fluorochromes.

from the natural limitations imposed by the specimen itself, resolution an image quality obtained in fluorescence microscopy depends on two factors:

image brightness, which is determined by the intensity of the excitin radiations;

image contrast, expressed as

$$\frac{\textit{intensity of object}-\textit{intensity of background}}{\textit{intensity of object}}$$

The latter is controlled by the light-condensing system employed to illumina the specimen and by light filters which may be incorporated in the optic system.

Two subsidiary but significant considerations are the light losses at th various air-to-glass surfaces in the microscope, and the degree of what known as 'veiling glare' in the system which reduces the contrast and tru colour values of the fluorescent image.

Filtration. Several commercially-manufactured light sources with suitab intensity and wavelength emission are adequate for most purposes (see Ligh Sources, p. 435). It is necessary, however, to select the wavelengths require by interposing suitable primary coloured liquid or glass filters between th source and the condensing system which only transmit light in the region re quired to excite fluorescence. A suitable secondary filter which absorb activating rays but transmits the fluorescent light emitted by the specimen ha

) be placed between the objective and the observer (often for convenience in ıe eyepiece). The filter prevents interference with the definition of the image ıd also protects the eyes from ultra-violet radiations that may enter the ıicroscope. Incorrect filter systems may be responsible for a considerable loss f brilliance and contrast in the final image.

Evaluation of these factors, as presented below, is based upon several years' ractical experience in biological applications of fluorescence microscopy. It as been possible to compare the advantages and shortcomings of bright-field ıd dark-field condenser systems and the different forms of light filtration hich must be used in conjunction with these, in relation to the study of ɔecific problems by some of the techniques in current use. In particular, :tention has been given to the need for obtaining a final image of high in-nsity with good resolution, contrast, and colour preservation, allowing ɔutine photomicrographic records to be made on colour film.

ractical systems for fluorescence microscopy

The bright-field condenser system. In this arrangement the substage optical stem is virtually that used for ordinary bright-field microscopy. If wave-ngths below 360 mμ were to be used to excite fluorescence, it would be ssential to employ a quartz condenser and lamp collector lens to ensure aximum transmission of ultra-violet light, but these shorter wavelengths are rely employed at the present time. Abbe and aplanatic design condensers are lequate for low and medium magnifications, but to obtain the best results, pecially when using immersion objectives, an achromatic condenser, NA 30 to 1·4, is recommended, immersed in oil to the undersurface of the slide ensure the maximum angle of activating light. In general the highest uminating aperture possible should be used, providing this covers the extent the field with the objective and eyepiece in use.

A proportion of the light, or all of it, depending on the ratio of the objective ɔerture to the focal length and aperture of the condenser, will pass through to the observing system of the microscope. Consequently with this system ıe whole waveband of activating light must be 'selectively' absorbed by eans of an appropriate secondary filter in the eyepiece of the microscope. ypical filter combinations recommended for general observation purposes in ıe study of tissue preparations are shown in table 1. The filters quoted are djusted to the intensity and spectral transmission of the 250-watt high essure mercury lamp. Sources with different characteristics will, of course, quire their own carefully-matched pairs of primary and secondary filters, ɔpropriate to the wavelengths required.

There are several theoretical disadvantages inherent in the bright-field ndenser system; these must always be considered if the equipment is to give sults which are acceptable from the optical standpoint and also biologically eaningful. The primary filter should, so far as possible, transmit only the avelengths needed for excitation of fluorescence, and the loss of intensity used by the denser filtrations required to achieve this end inevitably result

TABLE 1

Bright-field condenser

Primary filters			*Secondary filters*	
Stain technique	*Type*	*Maximum transmission*	*Type*	*Maximum absorption*
Primulin dye for distinguishing between living and dead cells	*Wratten No. 18A (glass)	365 mμ (52% transmission—ultra-violet excitation)	Wratten gelatin No. 3 plus No. 2B	at wavelengths shorter than 460 mμ
Fluorescein-labelled antibody (Coons technique)	*Wratten No. 50 (glass-mounted gelatin)	436 mμ (10% transmission—blue light excitation)	Wratten gelatin No. 8 plus No. 9	at wavelengths shorter than 500 mμ
Acridine orange nucleic acid staining method (Armstrong)	*Wratten No. 35 (glass-mounted gelatin)	436 mμ (37% transmission—blue light excitation)	Wratten gelatin No. 15G	at wavelengths shorter than 500 mμ

* A cell, 2 cm thick, containing a 10% copper sulphate solution, is always used in conjunction with these filters.

Note. The transmission of OX7 and Wratten No. 50 filters diminishes with continued exposure to ultra-violet radiation.

The selection of filters. It is advisable to check with a spectroscope the transmission of all filters used in conjunction with a light source that is suitable for the observations to be undertaken. Slight variations in density of the secondary filters will modify the fluorescent colours. Autofluorescent violet, blue, and white light emitted by activated unstained tissues, &c., may be appreciably altered in colour by certain filters that absorb ultra-violet and blue-violet light.

A correctly balanced colour range with blue light excitation should permit the unstained, non-fluorescent structures to be observed only as very weak blue dark-field images when employing induced fluorescence techniques.

An alternative filter combination giving an 80% transmission from 350 mμ to 410 mμ with the bright-field condenser is prepared by dissolving 0·75 g of iodine in 100 ml of carbon tetrachloride and using at 1 cm cell thickness for the primary filter. Secondary filters are Wratten No. 9 with Wratten No. 2B. This system cannot be used for blue and white light fluorescence emission.

Details of liquid and glass filters for the isolation of specific wavelengths of the mercury discharge lamp are given by Bowen (1946).

in diminished activation of the specimen. In addition, the need for a strongly absorbing secondary filter with fairly wide spectral absorption characteristics, results in a further sacrifice of intensity in the fluorescent image and also some degree of colour degradation. The latter may be serious, as interpretation of the fluorescent emission from a biological specimen may require an accurate identification and measurement and depend upon recognition by the observer of specific polychromatic effects, i.e. differential fluorescence.

The most serious light losses, however, occur at the specimen itself. Fluorescent light is emitted equally in all directions and it may be assumed that this is so with biological material as it is for a self-luminous body. Since objectives are used at full aperture in fluorescence microscopy the amount of light reaching the image plane, after refractions at the interfaces in the object plane, is primarily determined by the angle of the illuminating cone.

It will be seen from fig. 2, A that it is possible to utilize to advantage the maximum aperture of the bright-field condenser at shorter wavelengths (350 to 420 mμ). When the aperture of the objective is reduced in relation to the illuminating aperture, i.e. medium and low powers, the system becomes more efficient and is equivalent to using a combination of condensers, one giving a bright-field of NA equal to that of the objective and the other a dark-field system with a high aperture. The efficiency of this system depends on the low visible intensity of the light transmitted into the objective. A dry 4-mm objective of NA 0·95 will thus receive a 143·6° ($u = 71{\cdot}8°$) cone of ultra-violet radiations, as well as fluorescent light from the object, when illuminated with a condenser of NA 1·30 oiled to the specimen slide. If now an 8-mm objective of NA 0·45 is used with this condenser under the same conditions, the angular cone of rays received will be 53·4° ($u = 26{\cdot}7°$). Although the energy concentrated in the object plane will be the same as for the 4-mm objective, the proportion of activating light entering the 8-mm objective will be considerably less than half. Secondary filtration can therefore be proportionately reduced and an image intensity, relative to the illuminating aperture used with the 4-mm objective, maintained against a darker background. The ratio of light concentrated on the specimen to light received by the objective reaches unity when oil-immersion objectives of the same numerical aperture as the condenser are used. Filtration will then be at a maximum to absorb the high proportion of exciting radiations superimposed on the fluorescent image. Approximately 10 times as much light is collected by an objective of NA 1·30 as by one of NA 0·45 (fig. 2, B), and the degree of filtration necessary at any wavelength for these high-aperture objectives reduces the final image intensity to below that obtained with the dark-field condenser system. When the bright-field condenser is used for excitation of fluorescence with visible light it becomes even less efficient. Advantages of the objective/condenser aperture ratios, gained with ultra-violet illumination, are lost because of the high intensity of the background and the inability of the secondary filter to absorb these wavelengths completely without modifying the colour of the fluorescent image. In this instance the system is unsatisfactory when lower magnifications

are used since the intensity of the illuminating beams in the primary image plane will vary as the reciprocal of the square of the magnification and stronger secondary filtration will be required to absorb residual blue light.

Another complication, arising from the large illuminating apertures necessary to maintain a high fluorescence intensity, is an appreciable amount of 'veiling glare'. In the fluorescence microscope, unlike other microscope systems

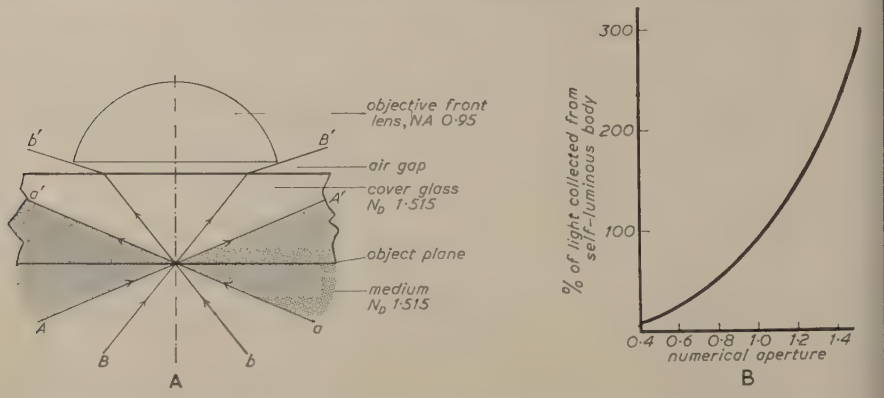

FIG. 2. A, section through the object plane, cover-glass, and objective front lens for a dry system. Illuminating cone for bright field condenser, *A*, *a*. Fluorescent light and direct radiations entering objective, B^1, b^1. B, relationship of the light concentrated on an object and the energy emitted by a self-luminous body to the light-gathering power of the objective. Calculation based on objective of NA 1·0 receiving 100% energy.

with the exception of the ultra-violet absorption microscope, glare can originate from the autofluorescence of glass lenses in the illuminating apparatus as well as from the specimen mount and lenses of the observing system. Apart from the presence of glare due to light reflections during visible light excitation, there is sometimes appreciable autofluorescence from glass slides, immersion oil, and lens components; this is most likely to occur when ultra-violet light is being used. Stray visible light from these various causes will mask the image of the specimen with a resulting loss in contrast and resolution. For these reasons, and also because of the low ultra-violet-transmitting properties of glass, it is advisable to employ a quartz condenser and collector lens when using wavelengths below 360 mμ to excite fluorescence. Tests have been carried out in this laboratory with glass and quartz lenses in the illuminating system. Results demonstrating two forms of veiling glare, often present in the fluorescence microscope, are shown in fig. 5. Total glare in a system originating from numerous causes may present a serious problem when the bright-field condenser is used and must be taken into account when assessing the performance of the microscope. Autofluorescence of the objective and eye-piece lenses can be eliminated by mounting a special ultra-violet absorbing filter on the front lens of the objective. Many of the yellow or minus blue filters that are available transmit a high proportion of the ultra-violet at 360 mμ and are quite unsuitable for this purpose or for the protection of the eyes. The

pectral qualities of these filters must be carefully checked before they are put into general use.

In spite of the various disadvantages associated with the bright-field system, experience has shown that in one respect it may have definite advantages over the alternative dark-field method. This applies to low-power microscopy (up to ×75), provided that excitation is by ultra-violet and not visible light wavelengths. With a well-corrected long focus bright-field condenser, having as high a numerical aperture as possible, extremely good results are attainable at these magnifications, whereas under the same circumstances the efficiency of the dark-field condenser system falls off markedly with the area of specimen requiring even illumination. There are instances when it may be necessary to examine or record large sections of material, e.g. in the study of naturally-occurring prophyrins in sections of bone and teeth, when only low magnifications (×20 to×30 diameters) are required. In these circumstances the bright-field condenser gives superior results at 360 mμ and is useful for rapid screening or counting of specimens in which fluorescence is activated by ultra-violet light.

Dark-field condenser systems. Since visibility is dependent on contrast it is desirable in fluorescence microscopy, when image brightness is often low, to aim for maximum image contrast. Dark-field illumination has proved superior in this respect to other methods precisely because it ensures a black background to the image, irrespective of whether ultra-violet or visible light is used for excitation. This is also true when incident annular oblique illumination is used for the study of the fluorescent surface structures of opaque specimens. The complications associated with the bright-field system are largely avoided, since all direct radiations from the light source pass from the top surface of the condenser outside the aperture of the objective lens and so cannot interfere with the formation of the fluorescent image. This also permits wide spectral bands for excitations, thus ensuring a high intensity level together with enhanced contrast.

The underlying principles are illustrated by a simple diagram (fig. 2, A). Here the larger angle *A*, *a* represents the entrance aperture to a bright-field condenser. By introducing a circular opaque stop centrally in this aperture, a hollow cone of light *A*, *a*, *B*, *b* will come to a focus in the object plane. Provided that the obstructed aperture of the condenser is larger than the objective aperture in use, direct light will not enter this lens and the object will appear bright on a black background. It is advisable to use the specially designed high-power dark-field condensers of the cardioid or bispherical forms for this purpose; these must always be immersed in oil to the undersurface of the slide. They can be used with all visible wavelengths, and down to 360 mμ in the ultra-violet, without loss of efficiency. They illuminate a field of sufficient size for use with both oil-immersion and dry objectives having focal lengths up to 8 mm, but the standard form of dark-field condenser is unsuitable for lower magnification than this. However, objectives of 16 mm (×10) focal length and ×10 eyepiece may be effectively used in the following way.

A special condenser for the purpose can be obtained by removing the front and intermediary lens components from an achromatic bright-field condenser and fitting a stop in the anterior focal plane, just large enough (i.e. one-tenth larger than the objective aperture) to prevent any direct light from entering the objective. Such a system gives results comparable in intensity and colour to those obtained with a 4-mm objective and cardioid condenser.

The dark-field condenser may be regarded, in this context, both as an illuminator and as an efficient primary filter, enabling the use of intense exciting radiations. Its efficiency depends largely on the relative refractive indices No/N_m of the structures in the specimen, where No is the index of the object and N_m that of the surrounding medium. When $No = N_m$ for a particular wavelength, no light of that wavelength will be scattered by the non-fluorescing parts of the object, and the image plane will be uniformly black. In fig. 3, A the object is represented by a black ink-mark. If the wavelength used can activate fluorescence in the specimen, only the light emitted by the specimen will enter the objective and reach the image plane. Clearly, an ideal specimen would be one in which the light dispersions of the object and its surrounding medium are equal, at least over the visible and long ultra-violet regions of the spectrum (fig. 4, B). A fluorescent object of this nature could then be activated by light from a selected source, no primary or secondary filters of any kind being needed. This was achieved by Barnard and Welch (1936) when they used monochromatic light at 275 mμ and a quartz dark-field condenser to record the autofluorescence of bacterial cell components.

A near approach to this ideal situation has been demonstrated in this laboratory, with fluorochromed specimens of rat spermatozoa (fig. 4, A, B). Smears were made on thin glass slides and fixed with formaldehyde-saline.

FIG. 3 (plate). A, dried black ink-spot in air without cover-glass. Excitation with ultra-violet at 365 mμ. Wratten 18A primary filter. Wratten 2B secondary filter. Condenser, cardioid. Exposure 1 min.

B, same preparation and conditions as A, but with bright-field illumination. Achromatic condenser NA 1·3. Exposure 1 min. Beck 4 mm. NA 0·95 apochromatic objective.

C, D, E, and F, section of cat-stomach fixed and stained with acridine orange No. 788, 1 in 2,000, and mounted in buffer (pH 2·7). Polychromatic fluorescence recorded in black and white with cardioid and bright-field condensers. Objective, Beck 4 mm. NA 0·95 apochromatic.

C, condenser, cardioid. Filters, primary, copper sulphate; and ammonia liquid; secondary, Wratten No. 4. Exposure 30 sec. Remarks: colour contrasts good between nuclei and cytoplasm. Contrasts good between stained and unstained tissues. Black background.

D, condenser, achromatic NA 1·30 oiled to slide. Filter and exposure as for C. Remarks: bright blue background with a high percentage of blue light masking image. General loss of colour and contrasts. Requires much stronger primary and secondary filtration.

E, condenser, cardioid. Filters, primary, Chance OX7; secondary, Wratten No. 2B. Exposure 1½ min. Remarks: Good contrasts maintained as in C. Fluorescence lower especially in nuclei owing to lower absorption of energy at these wavelengths.

F, condenser, achromatic NA 1·30 oiled to slide. Filters, primary, Wratten 18A; secondary, Wratten No. 4. Exposure 6 min. Remarks: lower non-visible wavelengths exciting fluorescence help to improve contrasts. Secondary filter absorbs more efficiently direct light reaching objective. Fluorescence intensity of nuclei very similar to E at these wavelengths. Contrast not as good as in E with some loss of colour purity and resolvable detail. Exposure 4 times as long as E.

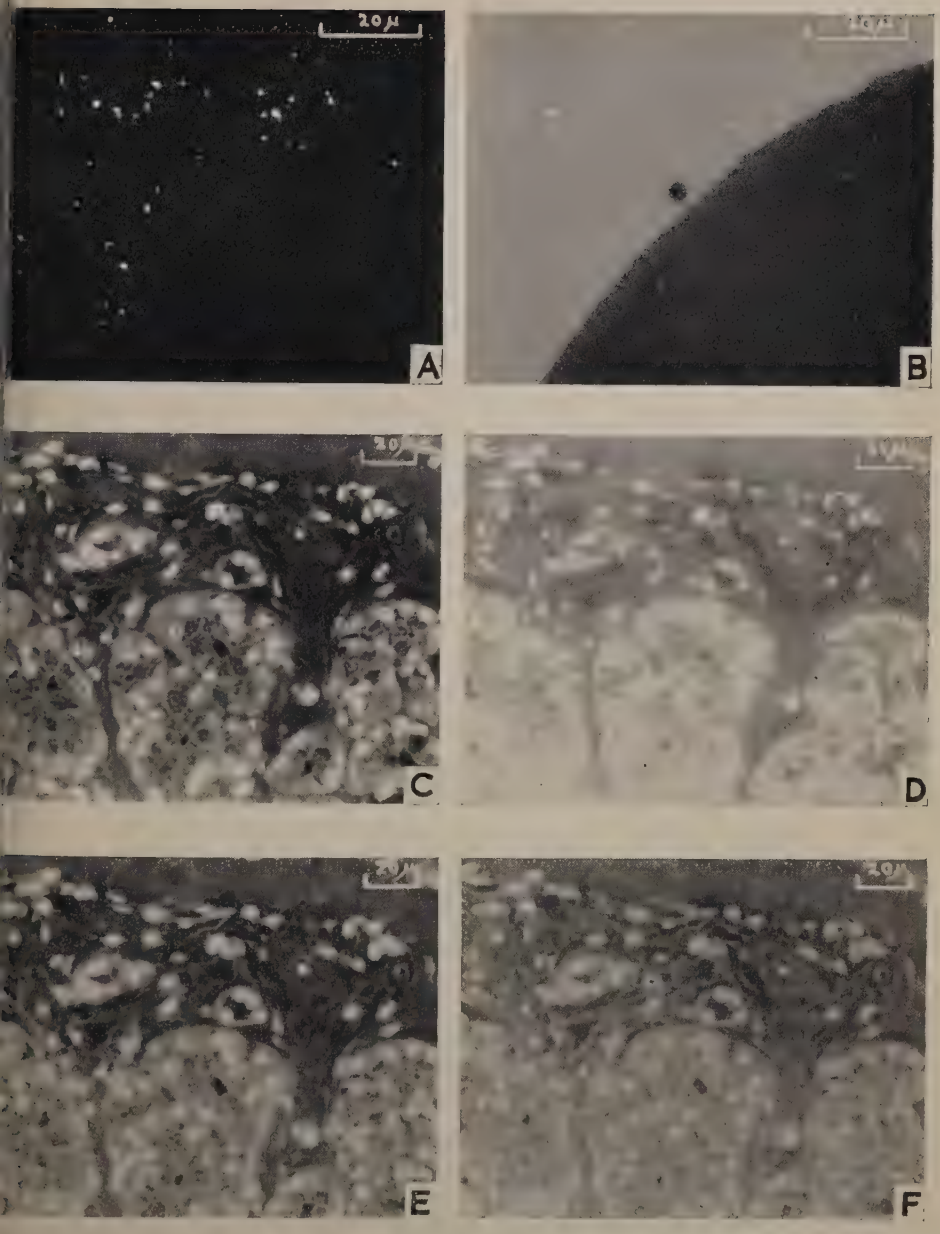

FIG. 3

M. R. YOUNG

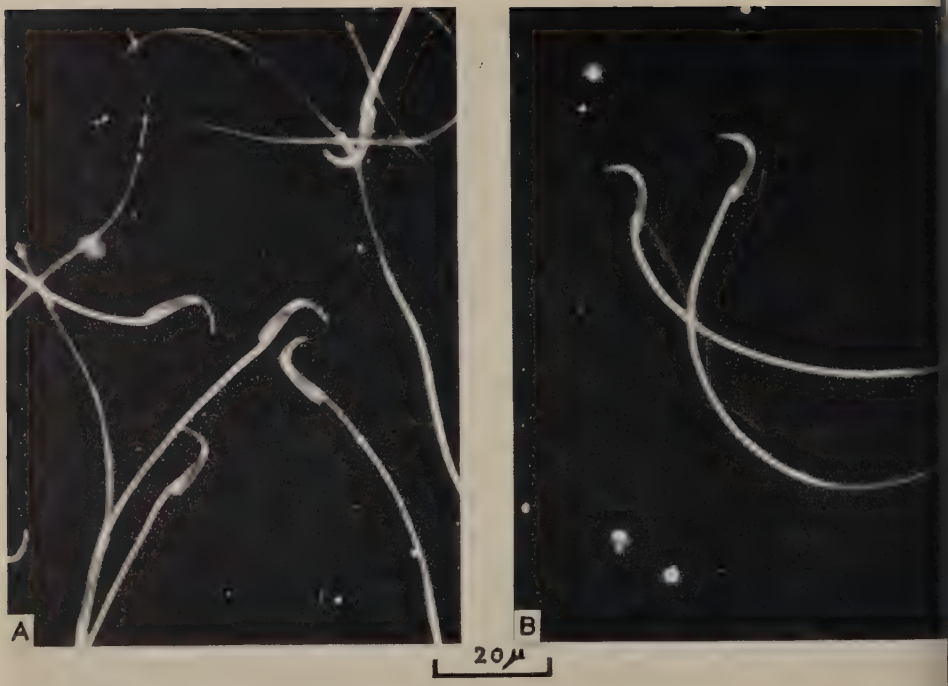

FIG. 4

M. R. YOUNG

After staining for 15 min in 1% rhodamine B (colour index 749), they were washed well in water, dried, and mounted in DPX ($N_D = 1{\cdot}524$) under a glass coverslip. The fluorescence microscope was fitted with a cardioid condenser and a dry objective (Beck 4-mm apochromatic, NA 0·95). The light source was a 250-watt mercury pressure lamp, and no primary or secondary filters were employed. Rhodamine B absorbs light energy strongly in the visible region, with a maximum at 550 mμ. Excellent fluorescent images were seen on a dark background. Where the cells had been stained with the dye the image had good contrast; but the unstained portions were perceptible as a dark-field image due to reflection into the objective of a small part of the incident light. It is inferred that the refractive indices of the medium and of the unstained parts of the cells were close but not identical under the conditions of the experiment. In general, as the difference between the object and medium increases more light is scattered, and the more obtrusive is the dark-field image which is superimposed on that due to fluorescence. This is the reverse of the mounting procedure for dark-field microscopy when resolution is dependent on refractive index differences between specimen and media.

Occasions when all filters can be dispensed with will rarely occur in work on biological specimens; for in fresh and in fixed materials the various cellular components usually differ sufficiently in refractive index to be depicted as a dark-field image. In practice, when ultra-violet wavelengths are required for excitation of fluorescence much of the light impinging on refractile non-fluorescent parts of the specimen will be scattered, and some of this will enter the objective lens. However, most of it is absorbed by the objective and eyepiece lenses and it does not harm the eyes nor interfere with the fluorescent image except in photographic records. Consequently no secondary filter is needed for direct observational work, while for photography it is enough to insert in the eyepiece an almost colourless ultra-violet absorbing filter such as the Wratten 2B. When excitation is with blue-violet or other visible wavelengths it becomes necessary of course to employ slightly stronger secondary filtration to absorb the visible dark-field image. Such filters range in density from the pale yellow Zeiss 'euphos' cover-glass type to the deeper coloured minus-blue filters familiar to photographers. Some selected primary and secondary filters, suitable for use with the mercury pressure lamp + dark-field

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Fig. 4 (plate). A, dark-field photomicrograph of a smear preparation of rat-sperm fixed and stained with 1% rhodamine B, photographed dry in air without a cover-glass.

B, fluorescence photomicrograph of fixed rat-sperm smear stained with 1% rhodamine B solution and mounted in DPX, N_D 1·524. Light source of A and B, 250-watt mercury pressure lamp. No primary or secondary filters used for either record. Condenser, Zeiss cardioid. Objective, Beck 4-mm NA 0·95 apochromatic with correction collar.

C, sample of living and dead spermatozoa stained with equal parts primulin at pH 7·2–8·2 (dye concentration approx. 1 : 30,000) and rhodamine 6G B.D.H. (dye concentration 1 : 30,000). The living spermatozoa fluoresce bright yellow and the dead ones light blue. Living spermatozoa will not absorb the primulin (after Bishop and Smiles, 1957). Primary filter, Chance OX7. Secondary filter, Wratten 2B. Excitation with ultra-violet light at 365 mμ. Condenser, Zeiss cardioid. Objective, Beck 4 mm NA 0·95.

condenser system, are given in table 2. Comparison of these filters with those given in table 1, in connexion with the bright-field condenser system, serves to illustrate the very different role of the secondary filters in the two systems of fluorescence microscopy. In the dark-field system it is less critical, and required only to absorb such light as may be scattered and reflected from the specimen into the observing system. It seems reasonable to suppose that if more tests were made of non-fluorescing mounting media with refractive indices similar to that of the specimen, the need for secondary filtration might be reduced even further in dark-field fluorescence microscopy.

Some additional factors of practical importance concerning primary filtration have been noted during routine use of the dark-field system in this laboratory. It is frequently necessary, when employing the fluorescein-labelled antibody technique (Coons and Kaplan, 1950), to differentiate conclusively between the specific apple-green fluorescence of cell structures binding the conjugate, and the non-specific autofluorescence and blue dark-ground image of non-fluorescing elements which are usually also present. For this technique excitation with the carbon arc or with mercury vapour lamp has proved satisfactory but the former emits a particularly intense spectrum over the entire blue-violet region. Some control on the intensity of the dark-field image can be a valuable aid for discerning the finer details of cell structure and the general anatomical relationships; on the other hand, as a check for the detection of very small amounts of the specific fluorescent dye it may be desirable to suppress the dark-field image below the limit of perception altogether. By increasing the transmission of the primary filter the more refractile elements can be revealed in greater detail, but with some loss of fluorescence visibility where take-up of the dye has been minimal. Conversely, curtailing transmission will diminish the brightness of non-fluorescent refractile elements and allow the weaker fluorescent details to be seen more clearly. For this purpose, in addition to a wide range of interchangeable glass filters, a variable thickness cuvette has been utilized to hold the coloured chemical solutions which can be used as an alternative form of primary filter with a useful range of transmission values.

Attempts have been made to improve the quality and intensity of the activating light, and trials carried out in this laboratory, with 'interference' primary filters, show that they have distinct possibilities, especially when it is desirable to select particular bands of the spectrum to obtain the specific maximum fluorescence of cell components. There are instances, however, with fluorochrome dyes, when high activating intensities are definitely harmful to the staining process and quality of the image. A 'saturation level' is reached and a severe lack of contrasts might result from this phenomenon. In these circumstances activating light of a lower intensity will excite the same level of fluorescence intensity in the specimen. Provided that the primary transmission is adjusted to this level, the degree of scattered light will be appreciably lower and a secondary filter with lower absorption can then be used.

Far more common effects due to intense excitation are 'photo-chemical'

TABLE 2

Dark-field condenser

Primary filters			*Secondary filters*	
Stain technique	*Type*	*Maximum transmission*	*Type*	*Maximum absorption*
Primulin dye for distinguishing between living and dead cells. Also suitable for autofluorescence	*Chance OX7 (glass)	240 to 400 mμ (87% transmission—ultra-violet excitation)	Wratten gelatin No. 2B	at wavelengths shorter than 420 mμ
Fluorescein-labelled antibody (Coons technique)	*Wratten No. 50 (glass-mounted gelatin)	436 mμ (10% transmission—blue light excitation)	Wratten gelatin † No. 8 or Ilford † No. 108	at wavelengths shorter than 460 mμ
and	or			
Acridine orange nucleic acid staining method (Armstrong)	Copper sulphate ammonia solution— $CuSO_4.5H_2O$ 25 g; NH_4OH(d = 0·88) 300 ml; dist. water 675 ml	400 to 500 mμ (60% transmission at 4-cm cell width—blue light excitation)	Wratten gelatin † No. 8 or Ilford † No. 110	at wavelengths shorter than 480 mμ

* A cell, 2 cm thick, containing a 10% copper sulphate, is always used in conjunction with these filters.

Note. † Autofluorescence of these filters is extremely low and does not interfere with quality of image. A 2B filter may be placed in front of the secondary filter to absorb stray ultra-violet light as a precaution.

changes and 'quenching'. These changes take place in both living and fixed tissues but are more rapid in the former, and may have to be controlled by reducing the intensity of the primary excitation. Overall intensity may be reduced by placing a neutral density wedge in the beam and moving it across the light path to a point where the dye will tolerate excitation without changing colour; alternatively the wavelengths of the exciting radiations may be adjusted to correspond with those absorbed by the lower slopes of the absorption curve of the dye. Primary and secondary filters would have to be selected for this purpose and observations made on lower intensity images. Radiation damage to living specimens is only noticeable after prolonged exposure to long-wave ultra-violet and blue light.

Measurements of the relative efficiency of bright- and dark-field condensers

Condensers used in the early days of fluorescence microscopy were of quartz, so that maximum transmission of the ultra-violet radiations was obtained. The field collector lens, liquid filter cells, and specimen slides were also of quartz for this reason. With improvements in the design of light sources the use of quartz lenses, slides, and cover-glasses now proves to be unnecessary with wavelengths normally employed to excite fluorescence. Absorption of ultra-violet at 360 mμ and at longer wavelengths by glass components is very small compared with the total light losses in the whole system.

In support of bright-field condensers Richards (1955) points out quite rightly that less light is concentrated on the specimen with the dark-field condenser. This is true when the condensers are used in the normal way for bright- and dark-field illumination. The difference, however, in the concentrating power is not as great as would appear. Ellinger (1940) considered the dark-field condenser less efficient because of the much smaller entrance pupil to the system. He failed to note that since the focal length of the bright-field condenser is greater than that of the dark-field, the light is focused over a greater area at a lower intensity.

Light intensity measurements were therefore made of the amount of energy concentrated on the object by each system. Köhler illumination was used and the field-iris reduced so that the area covered by a bright-field achromatic condenser, NA 1·30, was the same as that covered by a cardioid condenser. With a stabilized current for the light source and all other conditions being the same, it was found that the intensity for the bright-field condenser was 3 times as great under these conditions as for the dark-field system. A photo-electric cell (Mullard 90 A.V. vacuum cell) was used to obtain these results. The absolute fluorescent intensity of the image, with ideal filter systems for both types of condenser, cannot be determined accurately owing to the number of complex factors involved. By using a high sensitivity photo-multiplier it was possible to obtain comparative measurements for the two systems employing filter combinations recommended in tables 1 and 2.

A monolayer of tissue culture cells, stained with acridine orange, was chosen as a suitable specimen for the purpose of obtaining these readings. Since the

lye complexes formed in the cells absorb strongly in the ultra-violet and blue-iolet regions (see fig. 1), measurements could be made with the same speci-nen under conditions suitable to each of the condenser systems for these vavelengths. The specimen exhibited typically polychromatic fluorescence. The results obtained are shown in table 3. From these readings it will be seen hat the dark-field condenser shows an approximate increase in efficiency of .0% for the two bands of light used. Heavy filtration used for the bright-field ondenser to maintain a black background has greatly reduced its efficiency.

TABLE 3

	Bright-field condenser		*Dark-field condenser*	
Excitation wavelength	*Filters*	*Meter reading*	*Filters*	*Meter reading*
Jltra-violet 360 mμ	No. 18A plus No. 4	8	OX7 plus No. 3	11
lue violet 400–500 mμ	No. 50 plus Nos. 8 and 9	12	Copper sulphate and ammonia plus No. 8	17

urther, it was found to be even less efficient when accurate colour records re required, and to approach results obtained when using the dark-field con-lenser with blue light excitation of an acridine orange stained specimen, ltra-violet light had to be used (fig. 3). These results are confirmed by ex-osure times for several kinds of biological specimens stained with fluoro-hromes having different absorption characteristics. Light intensity measure-nents and the relative proportional values of energy collected from a self-uminous body are plotted against objective apertures and shown in fig. 2, B. he importance of separating the exciting radiations from the fluorescent mage-forming wavelengths at higher numerical apertures is demonstrated by his curve.

Photomicrographic records were also made to illustrate the background ntensity differences with ultra-violet and visible light radiations (fig. 3). hotomicrograph A shows the outline of an opaque ink-spot in air extending cross half the field. This was illuminated with intense ultra-violet radiations rom a dark-field condenser. Photomicrograph B is of the same opaque spot lluminated with a bright-field condenser. Other conditions were the same for oth condensers. The objective used was a 4-mm NA 0·95 apochromatic with orrection collar. Secondary filtration was not used for either system and the est spot was photographed dry without a cover-glass. As there are no efractile elements present in the test object the dark-field record should only how a black picture without any outline of the spot. There is some evidence f scattered light reaching the objective from the edge of the ink-spot and rom dust particles present in the field. Record B clearly shows the high in-ensity of the unabsorbed light reaching the objective. The remaining records, , D, E, and F, illustrate image quality and contrast obtained with the two

systems of illumination at different wavelengths. Identical secondary filtratio was used for C, D, and F. A colourless secondary filter, Wratten 2B, was use for recording E.

There is no general agreement about which type of condenser is mos efficient but it is evident from these results that several real advantages ar gained with dark-field illumination in fluorescence microscopy. The mos important of these is the maintenance of a black background to the specime when an intense band of exciting radiations is employed. The role of th secondary filter is simplified and quite different from that used with bright field illumination, serving only to absorb residual scattered activating ligh which is reflected into the objective by the specimen. Usually this is of a lo intensity and requires only weak secondary filtration. Owing to the inefficienc or lack of suitable light sources, the bright-field system requires exacting filte combinations with consequent losses of activating energy and deterioration o image contrasts.

In studies of vitally fluorochromed cells, photomicrographic records i colour of the fluorescent image are of the utmost importance (Bishop an Austin, 1957; Bishop and Smiles, 1957) (fig. 4, c); to avoid any changes takin place in the cell owing to ultra-violet or blue light radiations the exposures t light must be kept to a minimum. For this work the dark-field condenser ha proved superior to the bright-field system and recently it has been foun possible to detect and record in colour at 'high magnifications' various specie of acid-fast bacilli in tissue sections, stained with auramine and rhodamin (Kuper and May, 1960). The highest possible intensity of activating light i necessary for these critical observations and it is therefore necessary to us a very wide band of activating blue-violet light to excite maximum fluorescenc of these dyes with absorption peaks between 400 mμ and 556 mμ. At th highest magnifications with binocular vision ($\times$ 100 objective $\times$ 10 eyepiece the bacilli can be clearly observed fluorescing a bright golden yellow agains a blue dark-ground image of unstained tissue.

In assessing the relative merits of the two systems of illumination, n account has been taken of the relation of visual acuity to the brightness level compared. Observations should be made under as near normal conditions o lighting as can be comfortably tolerated. The brightness levels obtained wit the dark-field system enable one to use the microscope for long periods with out undue eye-strain. Differences of visual acuity from one person to anothe appear to be mostly associated with colour interpretation rather than bright ness level.

Use of polarized light. The practical application of polarized light to th fluorescence microscope to replace the usual filters has been investigated. Th specimen is illuminated with plane-polarized light of the required wave lengths, and an analyser, with its plane of vibration at right angles to th polarized beam, is placed between the objective and eyepiece. By this mean polarized activating light will be prevented from reaching the eye and onl fluorescent light from the specimen will be observed. Because of strong

lepolarization of light by the dark-field condenser this method of illumination as not been found suitable, but with the bright-field condenser polarized ght has certain advantages over ordinary light, especially in combination vith suitable filters permitting wide bands of the spectrum to be used to excite uorescence. There is no restriction to the numerical aperture that can be used nd glare is appreciably reduced. The most useful application of this method s to the study of polarized fluorescent light in relation to structure orientation. Nicol prisms must be used with the long ultra-violet, and objectives of up to < 20 (8 mm) with a ×6 eyepiece give good results. Image intensity at higher nagnifications than this is poor owing to light losses in the system. Prisms vith blue light are particularly useful at high magnifications but generally for nedium and low powers polaroid screens give better results.

Light Sources

The essential requirement for an appropriate light source is strong emission n the specific wavelengths required to activate fluorescence of the stained lements of the specimen which are to be studied. It is therefore necessary to onsider the emission spectrum of the source in relation to the absorption pectrum of the dye to be used. It is useful to have records available of the bsorption spectra, from the short wave ultra-violet into the visual spectrum, f the dyes being used for fluorescence microscopy (see fig. 2). Since the bsorbing wavelengths of the dye may be spread over a region of the spectrum, s is the case with fluorescein which absorbs from 460 mμ to 510 mμ, it will nen be necessary to consider the energy output of the source in this 'region' n relation to the remaining wavelengths emitted and the primary filtration to e used. It must be remembered that the absorption maxima may be shifted y formation of strong organic dye-complexes within the tissues.

The emission spectra and intrinsic brilliance of suitable lamps can be scertained from the manufacturers. At present there is no one source which vill satisfy all possible requirements of the microscopist.

Four types of light source are available for use with the fluorescence micro-cope, namely:

(1) High-tension spark-discharge between metal electrodes.
(2) Low- and high-intensity carbon arcs.
(3) Mercury discharge lamps.
(4) Tungsten filament lamps.

High-tension spark. In certain instances the high-tension spark will be ecessary if an intense source of energy is required in the ultra-violet region etween 200 and 300 mμ (Barnard and Welch, 1936). When it is important nat monochromatic radiation should be used to activate fluorescence it will e necessary to employ a monochromator consisting of two quartz prisms. his system will probably be of value in future investigations into micro-pectroscopic measurements of fluorescent compounds and complexes formed vith tissues (Acheson and Orzel, 1956).

Carbon arcs. The carbon arc is well known as a useful source of ultra-viole and blue light radiations. In the low-intensity arc energy is emitted over a wid range of the spectrum from 300 mμ into the infra-red; it has been widel favoured by many workers using the fluorescein-labelled antibody technique and since it has a high energy output over the whole of the absorbing region o the fluorescein dye, especially in the cyanogen bands at 415, 385, and 375· mμ, this source has proved very useful. Unfortunately, considerable energy i emitted at all other wavelengths, particularly in the red, which have to b removed by primary filtration, which greatly reduces the intensity of th exciting radiations. The efficiency of this type of source can be much im proved by shielding the electrodes from the collecting lenses. The tips of th electrodes are responsible for the longer wavelength radiations which are no required, and by selectively focusing on to the specimen only the rays emitte by the 'arc', primary filtration is simplified. In high-intensity arcs the positiv electrode consists of a carbon rod with a core of rare earth compounds an burns with a current density at the positive pole (D.C. current) about $3\frac{1}{2}$ time that of low-density arcs. All arc lamps correctly adjusted will provide a stead source of energy and should be operated by clockwork with suitable automati compensation for variations in the rate of burning. A real advance in th application of the carbon arc to fluorescence work could be achieved by design ing a suitably compact form of 'enclosed' arc, sometimes called a flame arc.

Mercury discharge lamps. Mercury vapour discharge lamps have prove suitable for most purposes in fluorescence microscopy. In view of the hig energy output which may be several times that of the carbon arc, and als their compactness and adaptability, these lamps are preferred in many lab oratories. In the ultra-violet the emission spectra have well-separated maxim at 312 mμ, 334 mμ, 365–6 mμ; a series of strong lines from 377 mμ to 408 mμ in the deep violet regions, and an isolated band with very strong emission a 435–6 mμ in the blue-violet. If a well-corrected collector lens is used to focu light from the arc, excluding any image of the electrodes, the red light emissio can be minimized. Residual red light and heat are completely absorbed by a acidified 10% solution of $CuSO_4$ incorporated in the primary filtration. Thi kind of lamp also emits strong lines in the yellow and green which ar sufficiently absorbed by primary filters which transmit up to 500 mμ. Mercur discharge lamps can be operated under varying conditions and degrees o pressure. The spectral energy distribution is determined by the operatin pressure which can be as low as 0·01 mm or as high as 20 to 30 atmospheres The main value of the vapour lamp lies in the intensity of irradiation in th blue-violet and ultra-violet regions at suitably separated wavelengths. Lamp that operate at higher pressure emit the maximum energy at longer wave lengths with a stronger continuous background spectrum than those workin at lower pressures; there is a relative fall in the intensity of the ultra-viole and heavier primary filters become necessary. Compact source lamps of 100 watts or more fall into this class and are mainly useful as blue-violet ligh sources. In certain instances when fluorescence is weak and the excitatio

avelengths are between 400 and 500 mμ, these lamps may have advantages over ıe lower pressure types. The most useful mercury lamp is the quartz mercury ischarge lamp operating at about 20 atmospheres and rated at 150 to 250 atts. Its output and intrinsic brilliance (25,000 candles/cm²) from an arc of or 3 mm length, remains steady for about 400 h and then drops by 40–50%. hese lamps have to be cooled after use before they will operate again and ıaximum brilliance is reached in 10 to 20 minutes. The mercury vapour lamp ın at atmospheric pressure has the most favourable spectral energy distribuon with practically no background and low emission at the longer wavengths. Unfortunately, it has not the intrinsic brilliance required for fluorscence microscopy.

Tungsten filament lamps. These are the least efficient type of source to use or any purpose in fluorescence work. The spectrum of the radiation emitted is f the continuous type and lies mostly in the visual and infra-red regions. lthough these lamps have been used as a source for exciting radiations in the lue region, a fair proportion of the energy responsible for fluorescence lies etween 450 mμ and 520 mμ. Workers claiming success with this lamp have sed fluorochromes with strong absorption in this region.

Observation System

Resolution. Provided that glare is reduced to a minimum and a completely lack background maintained, it is reasonable to expect, since all observations re carried out with the objective aperture fully utilized, a higher standard of esolution than is obtained with the ordinary light microscope (fig. 6, A). ecause the specimen is self-luminous each point on the object emits waves to ll the aperture of the lens and will be imaged by it to form similar points, the loseness of which will depend on the quality of the lens and its NA. This is in ccordance with the classical interpretation of resolution as formulated by bbe,

resolution $= 0{\cdot}61\lambda/\mathrm{NA}$, where λ is the wavelength of the light used to illuminate the specimen.

In fluorescence, λ represents the light emitted by the specimen, independent f illumination, and is usually in the visible region of the spectrum. All the ght originates from the specimen without any external rays taking part in the rmation of the image and each point of light emitted will be independent of ght coming from any other point. The image-forming rays are not therefore apable of interference as they are when an object is externally illuminated. here is always a possibility, however, of light rays from the source reaching ıe objective and interfering with fluorescent image-forming rays when right-field illumination is used. For these reasons when visible light is used is important to have an accurate filter combination if the highest possible esolution is required. By closing the aperture of the bright-field condenser to nprove the background and contrasts when high NA objectives are used,

further interference of the image-forming rays is caused and resolution is reduced.

Visibility. The dark-field system is of particular value when detecting light emitted from particles below the limit of resolution. It has been claimed by Levaditi and Panthier (1945) that there is no theoretical limit to the size of particles which can be detected. A notable advance employing new techniques has been made by Venetta (1959).

If it is desirable to obtain quantitative micro-intensity measurements when the fluorescence is below photo-emulsion sensitivity, a photo-image intensifying tube can be used to raise low energy levels to frequency contrast responses that can be recorded. An American instrument, named the 'astracon', has recently been developed and is capable of detecting single photons of light.

Objectives. For most purposes the usual achromatic objectives supplied with microscopes are suitable for fluorescence work. Some loss in image intensity is to be expected when oil-immersion apochromatic objectives are used, owing to the greater number of lens components present in these systems. There may also be a loss of image contrast with the older forms of these objectives because they contain fluorite lenses; this mineral has autofluorescent properties which will cause considerable glare in the image plane. The 4-mm × 40 apochromatic objective of NA 0·95 fitted with a correction collar permitting observations to be carried out on covered and uncovered specimens is, however, to be recommended, provided that it is reasonably new. It has the advantage over other objectives with a similar magnification of having a higher numerical aperture, yielding a much brighter image; it will be found most useful for general purposes as well as for photomicrography. Spherical and chromatic aberrations due to varying thicknesses of cover-glass and mountant above the specimen can be reduced to a minimum by proper adjustment of the correction collar. The presence of these aberrations can be easily detected, since isolated structures seen against a black background are surrounded by a diffuse halo. By rotating the objective collar a little at a time to reduce this effect and then refocusing the microscope, a position will be reached when the image is completely free from halo. Very high quality images are also obtained with the modern fluorite objectives. These give a greater depth of field at the equivalent numerical aperture to an apochromatic lens and with less glare because of the fewer lens components necessary for this objective. Images of good contrast with resolution almost equal to that of the apochromats are possible and they are quite suitable for colour photomicrography.

The water-immersion achromatic series of objectives are very useful, in particular the × 50, NA 1·0 with a working distance of 0·5 mm, when using dark-field illumination. Complete absence of any fluorescence of the distilled water used for immersing the lens is an additional advantage. Tests have been carried out with the flat-field objectives now available in the achromatic and apochromatic series. The dry objectives up to 4 mm, NA 0·65 give excellent images without any loss of intensity. Unfortunately the 'immersion' flat-field objectives are not suitable. There is an appreciable drop in image intensity

owing to the greater number of lens components necessary for this type of objective and no advantage will be gained by their use. Lenses coated against reflection give an image with slightly better contrast than ordinary lenses and this is particularly noticeable with immersion objectives.

Special objectives for fluorescence microscopy are available from certain manufacturers. These objectives have an ultra-violet-absorbing filter permanently mounted immediately in front of the lens system. This is the ideal position for such a filter but limits the range of secondary filtration. It is also important to make certain that these objectives can be used with covered specimens as well as uncovered temporary mounts. When it is necessary to use annular oblique illumination directed from above the specimen, e.g. opaque objects, to excite surface fluorescence, special objectives are necessary. Of these the ultropak and epi-illuminator series incorporating an incident dark-field system of illumination are to be recommended. Other reflecting systems have been found less efficient owing to the high proportion of exciting radiations absorbed by the optical components, causing a serious drop in image intensity. For intravital microscopy it is an advantage to use objectives fitted with special immersion caps and cones which enable one to maintain a focus below the surface of organs. Water or physiological saline are suitable immersion fluids to use for these observations. At medium and low powers with incident illumination there is an appreciable amount of light reflected back into the objective. Oblique lighting is therefore recommended but at higher magnifications light losses may be serious. Normal incident illumination will be necessary to maintain image brightness and good backgrounds are obtained in the ultra-violet. Blue light activation, because of strong reflections, requires an annular oblique system for all magnifications.

Image brightness obtainable from an objective of given NA at a certain fluorescence intensity level varies approximately as the reciprocal of the square of the magnification. It has been found in practice, therefore, better to use medium-power objectives with the highest permissible NA and an eyepiece magnification of $\times 8$ to $\times 12$, thus utilizing a maximum cone of fluorescent light to produce a high contrast image. This is possible with dark-field illumination up to apertures of approximately NA 1·0 with most biological specimens when mounted on slides 1 to 1·2 mm thick in a watery medium with refractive index of 1·33 to 1·45. As the refractive index of the mounting medium is increased the illuminating cone will become larger and more energy is concentrated on the specimen. Objectives of not more than NA 1·0 are therefore able to gain the advantage of the larger illuminating cone without altering the secondary filtration, since none of these rays enter the objective. Provided that the difference between the refractive indices of the specimen and the mounting medium is not too great, scattered light from the specimen, when excited with blue light, will not interfere with the image. Immersion objectives with numerical apertures up to 1·4 can be used to examine preparations excited with ultra-violet radiations at 365 mμ and only require a colourless Wratten 2B filter for secondary filtration. Beyond 400 mμ stronger filters are

necessary at full apertures. A point is reached with excitation by visible light when the intensity of the direct unobstructed beam entering the objective interferes with the contrast of the image, and it is necessary to reduce the objective aperture to maintain a black background. With correct secondary filtration apertures up to 1·30 NA have been used without any reduction in the image contrast.

Experiments were carried out with suitably stained sections mounted in paraffin oil on cover-glasses (of thickness 0·3 to 0·4 mm) in place of slides, and a Beck dark-field focusing condenser was used to illuminate the sections with blue light. Reducing the slide thickness permitted a more oblique cone of illuminating rays, enabling an objective of NA 1·25 to be used. No direct light entered the objective under these conditions. Image intensity was high against a black background with a marked improvement in the purity of the fluorescent colours. Condensers permitting an NA of 1·3 to 1·4 are not generally suitable for biological work because specimens have to be mounted in a medium of a refractive index higher than the maximum NA given by the illuminating beam to achieve a dark field. For general purposes, however, the dark-field system limits immersion objectives to an NA of 1·1 when visual light is used to excite fluorescence. The question arises whether the dark-field method can be applied to numerical apertures up to 1·4 NA. The only solution would be one in which full cone illumination is used with an objective having a central opaque stop mounted in it to obstruct the direct exciting beam of light. Such a stop would have to be of a diameter to allow the maximum amount of fluorescent light to reach the image plane. Calculation shows that the diameter must be of such a value that $\sin^2 U^1/2 = \sin^2 U/2$ or $\sin U^1/2 = \frac{1}{\sqrt{2}} \sin U/2$, where U is the angular aperture of the objective and U^1 the aperture of the illuminating cone of rays.

Immersion objectives are best fitted with an iris diaphragm rather than a funnel stop, to allow for the rapid adjustment of the lens aperture to the existing conditions. Care must be taken with the use of immersion fluids. With blue light illumination the objective maker's oil must be used to immerse the lens to the cover-slip. Ultra-violet excitation will require the use of a non-fluorescing oil and medicinal paraffin oil is quite satisfactory in this respect. Immersion of high aperture dark-field condensers to the underside of the specimen slide is always necessary whatever aperture objective is used.

Eyepieces. Most modern eyepieces of Huygenian and negative compensating design are quite satisfactory. When using fluorite or apochromatic objectives it is advisable to employ the eyepieces corrected for colour magnification as supplied by the manufacturer. It is a great advantage if the eyepiece lenses are 'bloomed'. Compensating and Kellner eyepieces of older design are not suitable owing to autofluorescence of cemented lens components present which causes a loss of intensity and considerable 'glare'.

Veiling glare. The presence of glare and its effect on the performance of the fluorescence microscope demands particular attention. In an optical system when visible light is used it is confined to the following causes: (*a*) 'mechanical

glare' arising from light reflected from mechanical and non-optical surfaces; (*b*) 'optical glare' produced by light scattered and reflected at and between air/glass surfaces of lens components and slide/cover-slip surfaces; and (*c*) 'specimen glare' due to particulate nature and variations in refractive index in the object plane, causing light to be scattered, diffracted, and reflected. These all contribute to stray light being distributed over the image plane, causing losses in contrast and resolution. In addition, the fluorescence microscope has a more serious form of glare due to the autofluorescence of the lens cements, lenses, and immersion oil in the system. When a bright-field condenser is used, this is transmitted into the microscope from the illuminating system where fluorescing cements, glass surfaces, dust, and grease all contribute to a much higher proportion of total glare in the microscope. Since dark-field illumination limits the primary cause of glare to the preparation, only scattered light in the object plane will produce autofluorescence of the lenses in the microscope, and this was subsequently found to be negligible. Tests were carried out to ascertain the degree of glare present employing (*a*) ultra-violet light, and (*b*) blue light radiations with the dark-field condenser. With radiations at 365 mμ, glare in the microscope was extremely low. Its existence mainly originated from the lens components of the compensating eyepiece of old design used in the test. When a quartz eyepiece was used in its place the glare was eliminated. With blue light a greater degree of glare was detected; it was largely due to unabsorbed activating light. Fluorescence photomicrographs illustrating the effect of glare on finely resolvable detail are shown in fig. 5.

Alignment of the Microscope

Accurate centration of the illuminating apparatus is, of course, necessary for good results in bright- and dark-field microscopy; it is even more essential in fluorescence work owing to the low light intensity levels which often have to be used, and maximum excitation is required. The system is sensitive to the slightest decentration which will drastically alter the fluorescence intensities, and colour photographs may reveal a serious degradation of the true colours. A simple method of setting up the light source and optical components can be adopted and is reliable for any method of microscopy with either the 'critical' or Köhler system of illumination.

The microscope. An optical bench 1 metre long, adapted for fluorescence microscopy, is illustrated in fig. 6, B. This apparatus has proved to be satisfactory for most requirements in fluorescence work and is easily adapted to other methods of observation. The bench should stand on a strong, heavy table to minimize vibrations for photomicrography.

The light source A is the 250-watt high-pressure mercury vapour lamp normally used for this work.

The collector lens is a Nelson type 2 lens aplanatic of $2\frac{1}{2}$-in. focal length.

The heat-absorbing chamber of water necessary when carbon and similar types of open arc are employed. This cell, of glass or perspex, requires to be $\frac{1}{2}$ in. thick to be efficient.

Two stands are required for the primary filters. The first holds glass cells of different thicknesses to contain liquid filters; the second supports a sliding metal frame taking two glass filters, 50 mm × 50 mm × 6 mm, permitting a quick change from one filter to another during examination. The microscope is mounted on a special base-plate screwed to a broad saddle.

Metal shields are provided to screen off any stray light from the lamp, and with a correctly balanced filtration system it is perfectly satisfactory to work in a darkened room with a table standard and 75-watt lamp at 4 ft behind the observer. By dispensing with the microscope mirror and observing with the body-tube in the horizontal position, an appreciable gain in intensity from the light source is achieved, especially with wavelengths from 300 to 400 mμ.

When bench space is limited and it is necessary to use the microscope for general purposes, an optical bench half a metre long will be more convenient. The microscope is then placed in the upright position at a suitable height on the laboratory bench, so that the axis from the light source to the centre of the mirror can be easily established. By substituting the primary fluorescence filters with neutral density screens or visible light filters the microscope can be converted to other methods of observation, e.g. bright-field, phase contrast, dark-ground, &c. To be able to do this quickly without any further adjustment to the illuminating system is of the greatest value in fluorescence studies as it enables the observer to identify localized fluorescent areas or inclusions in terms of the general morphology of the tissues. Directly comparable fluorescence and bright-field observations can then be made. A neutral density screen is necessary in addition to a green filter (Wratten No. 61) to observe the specimen by dark-ground. Fluorescence studies of motile organisms and observation of any changes that may take place owing to irradiation and reaction to staining are easily made with this system. Small changes in refractility are also easily seen (Bishop and Smiles, 1957).

When arc lamps are used, it is necessary to adopt the Köhler system of illumination because of the shape and instability of the light source. With

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Fig. 5 (plate). Examples of veiling glare.

A, the specimen was a monolayer of normal pig kidney-cells stained with acridine orange and photographed on Ilford Pan F 35-mm film. Condenser system, bright-field C. T. & S. achromatic condenser NA 1·30; maximum excitation wavelength, 360 mμ; Beck 4-mm apochromatic objective, dry.

B, same field as A, photographed under identical conditions with the exception of the condenser which was replaced with a quartz system. There is a perceptible loss of contrasts and background density in A caused by the autofluorescence of the glass components at this wavelength.

C, a specimen similar to A. Cells photographed on Ilford Pan F 35-mm film. Condenser, Zeiss dark-field cardioid. Objective, Leitz 2 mm. Immersion oil used, maker's oil supplied for use with this objective at ordinary wavelengths (N_D 1·520). Maximum excitation wavelength at 350 mμ.

D, taken under the same conditions as C with the exception of the immersion oil. Non-fluorescing medicinal paraffin oil was used. The obvious loss of resolution and contrasts in C when compared with D are due to the strong autofluorescent properties of the immersion oil used under these conditions.

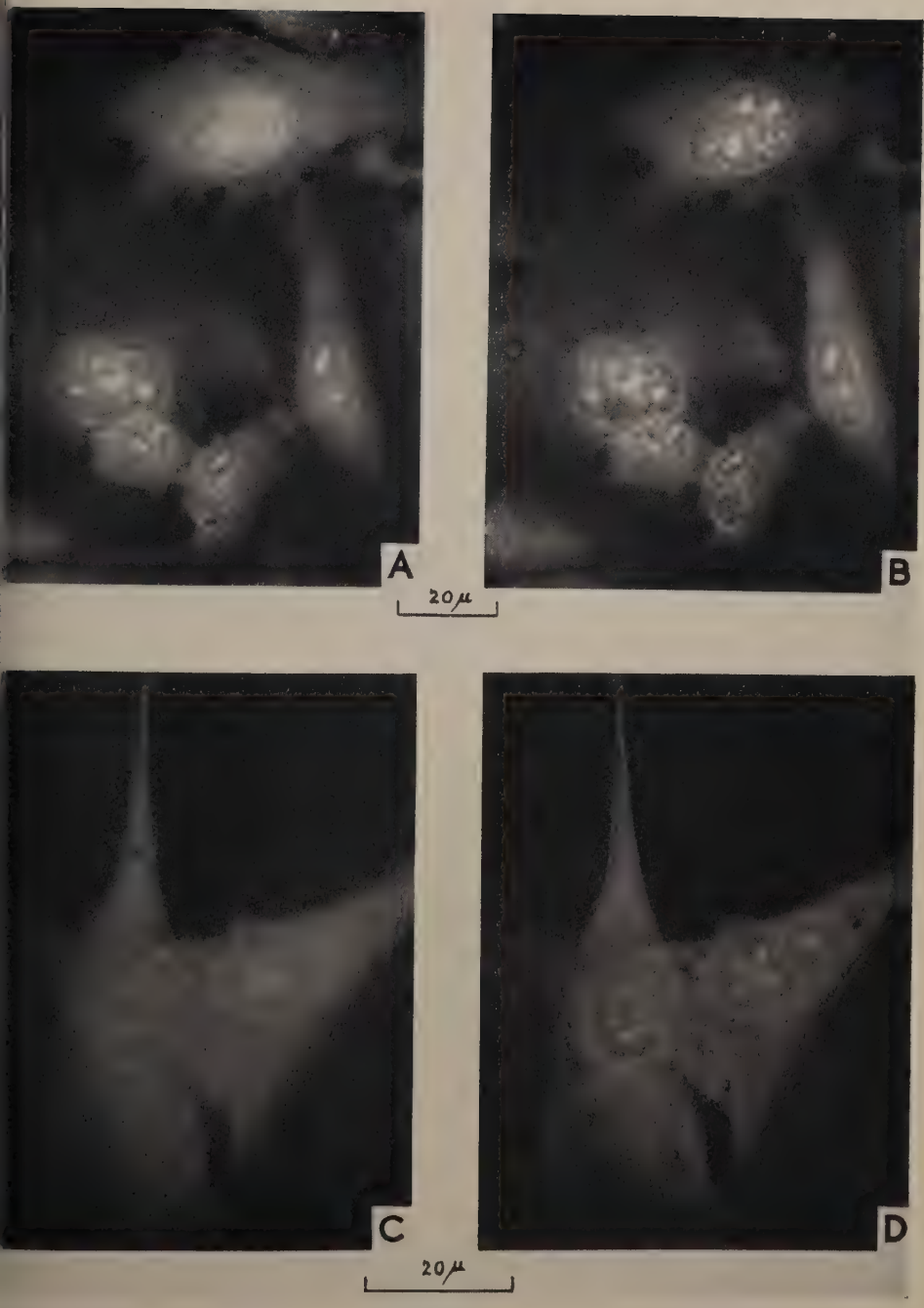

FIG. 5

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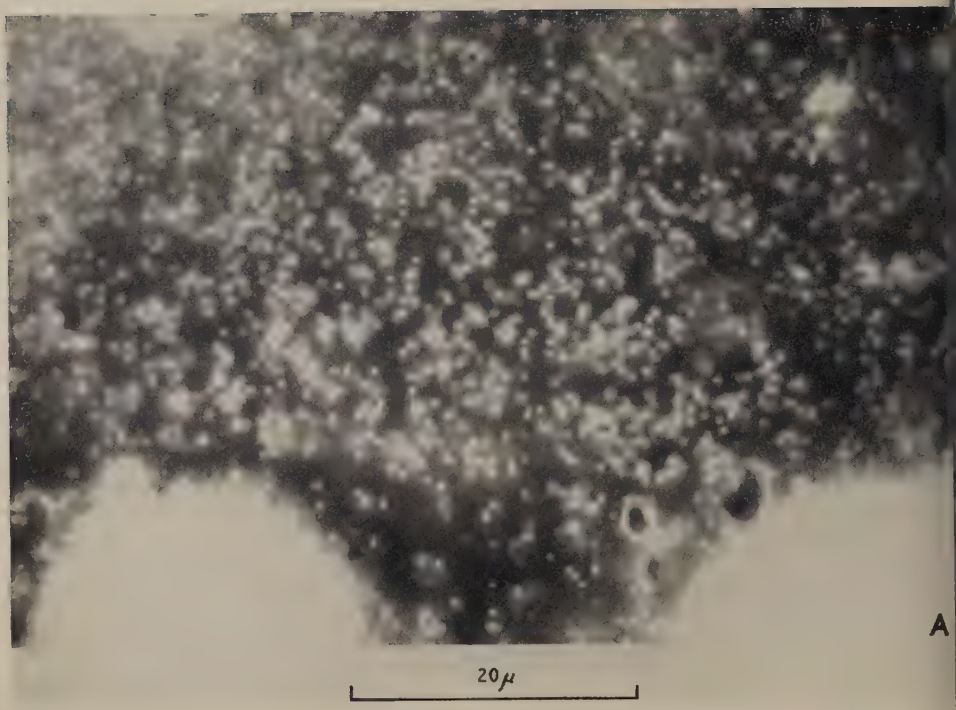

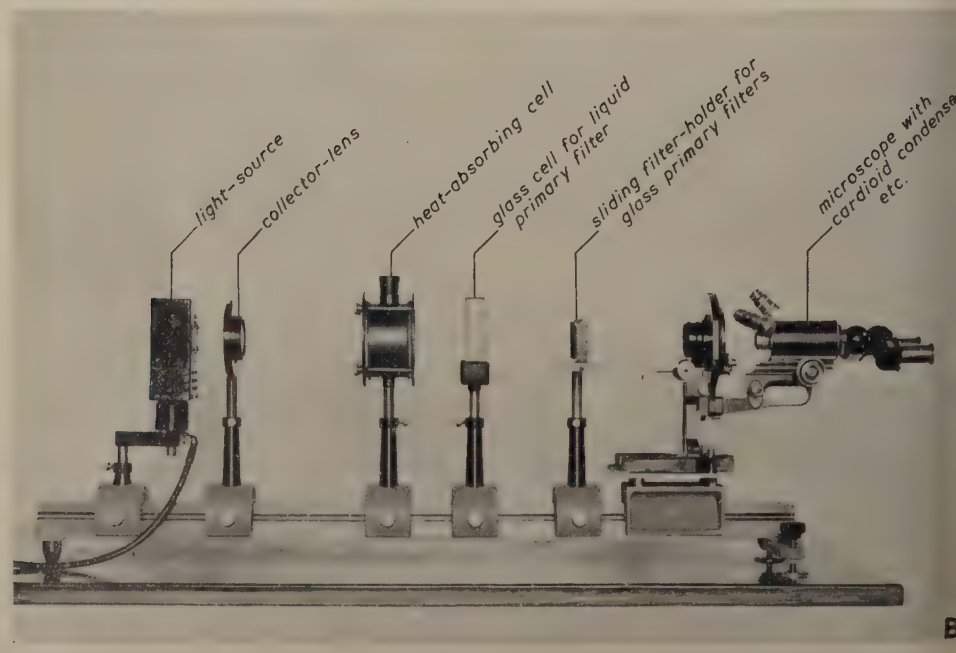

FIG. 6

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oil-immersion objectives of the highest power and eyepieces of medium power (×10 to ×12), it is possible to use 'critical' illumination with this type of source; but it is most important that the field of observation should be evenly illuminated. This is not always possible owing to the fact that the arc is focused in the plane of the object and vignetting at the edges of the field is more noticeable in photographs than by observation. Köhler illumination overcomes these difficulties and ensures an even field of illumination. This is achieved by focusing the condenser on a point slightly in front of the field collector lens and imaging this plane on the object when viewed through the microscope. An iris diaphragm is usually placed in front of the collector lens for this purpose and also for control of the area of the field of illumination.

Alignment with microscope in vertical position. For microscopes of modern design, with inclined binocular head, it is more convenient for the instrument to be set up in the vertical position and if possible mounted on a base-plate attached to the optical bench. Allowance must be made for the height of the instrument, when correctly aligned with the illumination, for comfortable observations to be made. When ultra-violet light is used and the fluorescence intensity is low it will be advantageous to have the surface of the microscope mirror silvered to give the maximum transmission at these wavelengths. It is essential that the light source, collector lens, and microscope lenses should be brought into coaxial relationship with the axis of the body-tube of the microscope. To do this it is necessary to ascertain the axis for these components in the following manner.

1. Remove condenser, objective, and eyepieces from the microscope. Adjust the position of the optical bench in front of the microscope with the single levelling screw, at one end, nearest to the mirror. The microscope should stand with the mirror approximately level with the light source when clamped in position ready for use.

2. Place two short stem saddles, one at each end of the bench (see fig. 7), and clamp firmly with retaining screws. Set the first alignment rod in the stem of the saddle as illustrated, with the point approximately the same height from the bench as the centre of the light source will be when mounted in position. Set the second alignment rod in the second saddle nearest the mirror at exactly the same height as the first rod. It may be necessary to incline the optical bench towards the mirror, roughly aligning the points of the two rods with the centre of the mirror.

3. Place a pinhole eyepiece in the body-tube and look down the microscope; tilt the plane surface of the mirror to direct an image, by reflection of the

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FIG. 6 (plate). A, smear preparation made on a glass slide from the freshly cut surface of a tumour-like lesion in the skin of a Rhesus monkey. Stained with acridine orange at pH 2·7. The lesion was produced by inoculation with a filterable agent allied to the pox viruses. Two cells in the lower part of the field are breaking down to release clusters of elementary bodies, which emit the greenish yellow fluorescence of DNA-containing structures. Diameter of the elementary bodies = 250 mμ approx. (measured with the electron microscope).
B, the fluorescence microscope. (The field iris is not shown.)

alignment rods, up the body-tube. Adjust by raising or lowering the first roc with the levelling screws of the bench until the tip is exactly behind and leve with the tip of the second and viewed centrally in the nosepiece aperture. The axis $A\ A^1$ (fig. 7) is now established and will be coaxial with the axis of the body-tube. The position of the mirror is now fixed and must not be moved.

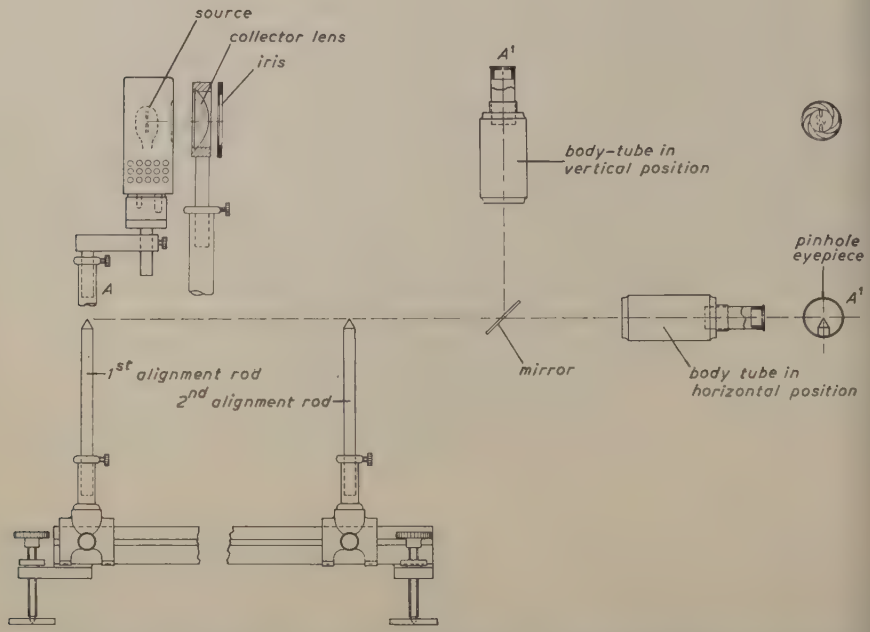

FIG. 7. Diagram of optical bench with alignment rods in position for setting up the microscope in the horizontal and vertical positions. A, A^1, optical axis.

4. Remove the first alignment rod. Place the light source and filter stands on the bench as shown in fig. 6, B. Switch on the source and with a suitable neutral screen in the second filter stand, observe the point of the second alignment rod through the pinhole eyepiece. Adjust the height of the source with the two levelling screws of the bench so that it is centrally aligned with the point of the second rod. Remove this rod and place the collector lens in position in front of the lamp; focus a parallel beam on to the mirror. Observe the image of the source through the pinhole and bring it central in the nosepiece aperture by centring the collector lens with centring screws.

5. Mount the condenser, a low-power objective (16 mm), and eyepiece on the microscope. With a suitable specimen (a section of tissue will do) on the stage, immerse the top lens of the condenser to the lower side of the slide with non-fluorescent immersion oil. Adjust the collector lens so that the beam just fills the entrance pupil of the condenser and then close the field iris diaphragm. Focus a sharp image of the diaphragm in the plane of the object by carefully

ıising the condenser and finally centre the diaphragm in the field with the ɔndenser centring screws. Open the field diaphragm fully and with suitable rimary and secondary filters in position the microscope is ready for fluor- scence observations.

It will be necessary to readjust the focus of the condenser for any variations ı slide thickness when changing specimens. Centration of the condenser may e required when changing from one objective to another and should always e checked before making any photomicrographs. To obtain a maximum area f illumination the source and collector lens should be moved closer to the ıirror until no further increase in size of the field diaphragm is observed when losed. It should not be necessary to recentre the collector lens if the alignment ; carried out accurately. The specimen should always be shielded from the ght source when observations are not in progress and searching time kept to minimum because of the rapid fading and quenching of many fluorochromes vhen excited with ultra-violet and blue light.

Alignment with microscope in horizontal position. In this arrangement, which ; to be recommended for research purposes, the instrumental adjustments are implified by omission of the mirror, and the microscope become an integral ›art of the optical bench. The height of the microscope body-tube from the ›ench will be determined by the type of light source to be used and it is ad- isable to have an adjustable base-plate made to fit into a strong saddle for the nicroscope to stand on (fig. 6, B). Since the body-tube axis in the horizontal ›osition may not be exactly parallel with the optical bench, it is important to stablish a common axis from the centre of the source to the eyepiece of the nicroscope. It will only be necessary to re-establish this axis when the light ource is changed. The procedure is as follows.

1. Ensure that the microscope is clamped firmly in position on the bench vith the body-tube aligned with the optical bench when fully in the horizontal ›osition. Remove the condenser, objective, and eyepiece.

2. Clamp a saddle and the first alignment rod to the opposite end of he bench. Place a pinhole eyepiece in the microscope and adjust the height of he tip of the rod, when viewed through the eyepiece, so that it is in the centre ›f the body-tube nosepiece aperture. Clamp a second saddle and second rod nidway between the first rod and the microscope. By viewing the tip of the irst rod, place the tip of the second rod so that it is exactly central in the nosepiece aperture and aligned with the tip of the first. The axis $A\,A^1$ is now stablished. A sheet of white paper held behind the rods will aid in sighting the ips.

3. Remove the first rod and mount the light source at approximately the ame height from the bench in its place. Observe the second rod and the ource through the pinhole eyepiece; adjust the height and lateral position of he centre of the source so as to be directly behind the tip of the second od.

4. Remove the second rod and place the collector lens and filter stands in ›osition on the bench. Focus the collector lens so that the nosepiece is filled

with light. Observe the image of the source (with a dense neutral screen in the filter stand) through the pinhole and bring it central in the nosepiece aperture with the centring adjustment of the collector lens.

Finally carry out the adjustments described in section 5, p. 444. Make a quick check for centration while the microscope is in use, by replacing the specimen with a slide coated with fluorescein to which has been added a proportion of gelatin to form an emulsion. This is allowed to dry on the slide, covered, and sealed. The coating forms a satisfactory screen for observing the image of the field diaphragm with a low-power objective. A thin coating of uranium nitrate crystals is also a useful test object.

Alignment with incident light. To centre a system with normal incident light, i.e. full aperture lighting from above the specimen, or with annular incident oblique light such as is used in the ultropak and epi-illuminators, the same procedure is carried out as outlined for substage illumination. This may not be possible with 'built-in' systems of modern microscopes.

To observe an image of the source with the incident illuminator screwed into position on the body-tube but without objectives, &c., in position (see stage 1 above), place a 3 in. × 1 in. slide (the upper surface of which is silvered) on the stage directly beneath the illuminator. With pinhole eyepiece in position an image of the source can now be clearly viewed. The axis of the illuminating beams from the source should be at 90° to the body-tube axis in the vertical plane and centred on the entrance aperture of the illuminator before assembling the objective. The silvered slide is then removed and a specimen put in its place. With an objective and eyepiece in position the stage is carefully raised to the focus of the objective by means of the stage rack adjustment. The field-diaphragm is adjusted to the same plane of focus as the specimen. Any lateral movement of the field-diaphragm image on the specimen on focusing indicates decentration in the system.

Preparation of Specimens

The specimen and mountant. Observations can be made upon either fresh material or semi-permanent preparations. Unless the specimen can be stored in the dry unmounted state, e.g. crystals, fibres, or fixed smears and films, &c., it is not usually feasible to preserve mounted specimens indefinitely. Satisfactory semi-permanent preparations of stained sections or monolayer tissue cultures can be made by mounting in suitably buffered aqueous solutions, glycerol, or physiological saline. For the study of certain types of biological material blood-serum is a most useful mountant. Observations on blood-parasites are often advantageously made by mounting the specimen in serum taken from the host, which must, of course, be fresh. After a few days serum exhibits a strong bluish-white autofluorescence.

The mounted specimens are sealed with wax (2 parts beeswax, 1 part dental wax) and will often retain their fluorescent qualities for several months without deterioration, especially if stored in the dark. Dry specimens, and in certain

nstances fluorochromed sections, may be mounted to advantage in castor or paraffin oils (medicinal). These have only a small amount of autofluorescence at 365 mμ. The most suitable medium for permanent mounts is DPX (N_D 1·524). It has a low autofluorescence and will preserve the fluorochrome dyes over long periods (when a neutral pH is maintained). This medium can only be used with dyes that are not removed by alcohol and similar solvents, which in practice is a serious limitation. If blue light is to be used to excite fluorescence in the specimen, Gurr's fluoremount, Apáthy's medium, or a solution of hyrax (Flatters and Garnett) have also proved successful.

Loss of image contrast can be due to the presence of excess dye which has diffused out from the specimen to form a uniform fluorescent background. This is avoided by adequate washing of the specimen in water or buffered solution before mounting, but specimens can often be washed and remounted if necessary. Saline has been used very effectively to clean away excess dye in old specimens stained with fluorescein-antibody conjugate. These will retain their fluorescence longer if stored at 4° C. Perfection of the image will depend on the thickness of the specimen in relation to the depth of field of the objective in the object space. To obtain images of good colour-contrast the thickness of the specimen should not be more than twice the focal depth of the objective in the object space and should depend on the morphological distribution of the fluorescing structures of the object. For the best results paraffin and freshly cut sections of tissue should be thin, certainly not more than 3 μ thick for higher aperture work. Similar advantages are gained by limiting the optical path between the undersurface of the cover-glass and the specimen. Monolayer cell cultures on cover-glasses are ideal specimens for fluorescence microscopy. Smears and films are best made on the cover-glass and mounted on to the slide so as to eliminate the layer of mountant between the specimen and cover-glass. Only the smallest quantity of mounting medium necessary should be used. Freshly cut frozen sections of tissue are best mounted in glycerol, but if allowed to dry on the slide, paraffin oil has proved a good mountant. Slides coated with agar (2% or 3%, diluted with an equal volume of buffer or serum) have proved extremely useful for mounting living tissues, &c., and enable a clean background to be obtained with the cells spread evenly with just enough pressure from the cover-glass to reveal details of structure. Provided that the coating is sufficiently thin (0·5 to 1·0 mm), light scatter and autofluorescence of the agar are not noticeable. Certain kinds of specimen are best examined dry and this may be done with the specimen uncovered. Bone and tooth sections must be ground and polished. Sections of natural bone can be prepared down to 50 mμ thick and must be examined with the bright-field system owing to scattering of light at wide angles of illumination with dark field. Sections of teeth 25 mμ thick give excellent images by dark field. Before examination with higher-aperture dry objectives, corrections must be made with the correction collar to compensate for the absence of a cover-glass. With refractile specimens, scattered visible light transmitted by the primary filter (red light with the 0×7 filter, unless a solution of copper sulphate is used)

will be reflected into the objective. In this case the specimen must be examined, covered and mounted in a medium of suitable refractive index. Freshly cut frozen sections have very little autofluorescence, but after storage the autofluorescence increases in intensity and a stage will be reached when this may interfere with the colour and strength of any dyes applied to the tissues. For these reasons specimens are best examined, and photographed if necessary, soon after mounting. Studies of autofluorescence must be made with fresh material and precautions taken to preserve the neutral reaction of tissue without extraction of the fluorescent substances during the process of mounting.

Slides and cover-glasses. When the cardioid dark-field condenser is used the thickness of the slides must not exceed 1·2 mm and when the microscope is used in the horizontal position should not be less than 1·0 mm thick. It is necessary to know the cover-glass thickness when high-aperture dry objectives, not fitted with a correction collar, are used. Only those that are 0·16 to 0·18 mm thick should be used. Slides must be chemically clean and free from grease to maintain the true fluorescence colours and image contrast. Certain batches of slides and cover-slips, even after cleaning by the usual methods, take up the basic fluorochrome dyes when used in the normal way for fluorescence microscopy. These may fluoresce quite strongly and produce a coloured background, which reduces contrast and detail in the image. The take-up of the dye is due to an almost insoluble film on the surface of the glass, which can only be effectively removed by polishing. By applying a wet polishing powder of alumina or a commercial glass cleaner, and polishing with the flat end of a wooden rod to which is attached a piece of 'selvyt' cloth, the contaminating film can be removed quite easily. The slides and covers should then be rinsed in several baths of hot water and dried. It is advisable to check each batch of slides and covers for dye 'take-up' before resorting to special cleaning methods.

Only when fluorescence is low and a quartz condenser is used with excitation wavelengths at 365 mμ or below is it necessary to resort to quartz slide and cover-slip preparations. The majority of slides supplied transmit freely down to 350 mμ and any autofluorescence of the glass has been found to be negligible for most purposes. Effective secondary filtration of ultra-violet light for temporary mounts in water, buffered solutions, glycerol, &c., is obtained with ultra-violet-absorbing cover-glass (Zeiss 'euplus' covers). These are used in place of ordinary cover-glasses and absorb strongly wavelengths up to 400 mμ and transmit 80% of the remaining spectrum.

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A New Automatic Machine for staining Histological Sections

By S. DILWORTH

(*From the Wool Industries Research Association, Torridon, Headingly, Leeds* 6)

With one plate (fig. 1)

Summary

A machine is described for the automatic staining of sections of tissue, mounted on nicroscope slides. It is narrow and compact, and can be placed along the back of a ɔench or on a shelf. Although for the present purpose the machine is designed to ccommodate up to 26 tanks, any number less than this may be used. A rack holding o slides is attached to a carrier, which then conveys it through the various solutions, ccording to a predetermined programme.

Introduction

THE machine described in this paper has been designed for the routine staining of large numbers of sections of tissue. It was designed for a ompound stain used for examination of sections of sheep-skin for studies of ool-growth. The stain, known as *sacpic*, was described by Auber (1950–1). t requires 26 stages, including the removal of the wax used as an embedding nedium, and various alcohols for up- and down-grading and differentiation.

For the work being done in the laboratories of the Wool Industries Research Association, it is necessary to stain a large number of sections in a outine manner to produce preparations that are as much standardized as ossible. Staining had previously been done by hand; racks carrying 10 slides ere taken through a series of staining jars, the whole process taking about $\frac{1}{2}$ h. Variations in the intensity of the stains resulted from inaccuracies in ming the various stages; the need, therefore, was for a method which would roduce evenly stained sections by accurate timing, and would also relieve ssistants for other work. Existing machines were examined but these were not atisfactory for this purpose, as they did not make provision for the number of tages required by the staining method.

Description of the Machine

The stainless-steel tanks, containing the various solutions, are placed in orrect order, between guide-rails and end-clamps, on the baseboard of the achine (fig. 1). Situated to the left of the first tank is a headstock bracket igs. 1, 2), on the nearside of which is mounted a bearing (fig. 2), carrying a ng slotted tube, located endwise, at a suitable height above, and extending eyond, the row of tanks. The other end of the tube is supported in a bearing ig. 3) on a tailstock bracket (figs. 1, 3), to the right of the tanks.

Quarterly Journal of Microscopical Science, Vol. 102, part 4, pp. 451–454, 1961.]

Running down the centre of the tube and splined into a sleeve at the tailstock end, is a shaft (fig. 3), notched at intervals corresponding to the tank spacings. A compression spring (fig. 4) inside the slotted tube presses on the shaft end from the tailstock position. This maintains engagement of the opposite end. It projects beyond the headstock bearing and engages with a double-lobed cam (fig. 2, *c*), fixed to the output shaft of a geared motor (fig. 2) mounted on the headstock bracket. Rotation of this cam reciprocates the shaft inside the tube. The tube and shaft can be rotated together by means of a reversible geared motor (fig. 3), mounted on the tailstock bracket.

The slotted tube carries a pulley (fig. 3). A spring-loaded pawl (fig. 3) is hinged to the pulley face. This pawl engages the notched shaft through the slot. It will, therefore, be seen that if the pulley is placed immediately above a tank, with its pawl engaging a notch in the shaft, and the cam is rotated, to push the shaft forward inside the tube, the pulley will slide along the outside of the tube through a distance equal to the throw of the cam, and the pitch of the notches. In this way the slide-holder (fig. 3) is moved from tank to tank.

The pulley is flanged to locate a nylon ribbon, one end of which is fastened to it. The ribbon passes around the pulley and has a hook attached to its free end, on which the slide-holder is hung. Rotation of the tube in one direction or the other raises or lowers the holder.

The timing cycle is controlled by a Sangamo geared motor (fig. 2, *d*), driving a Tufnol disk (fig. 2) at a speed of 1 revolution in 3 h. The arm of a micro-switch (fig. 2) rides on the edge of the disk, certain sections of which have been removed by means of a special punch. The rise and fall of the arm on the stepped edge sets in motion the indexing mechanism for moving the slide-holder. The sections in between the steps represent the dwell-periods in the tanks. The disk is calibrated, so that a pointer (fig. 2) on the arm of the micro-switch indicates the starting position for the slide-holder, and, as the disk rotates, it also indicates the tank in which the slides are immersed, and the duration of immersion.

The sequence when the micro-switch operates is as follows. The reversing motor (fig. 3) starts up, rotates the slotted tube and pulley, and raises the slide-holder out of the tank. After a suitable pause to allow for draining, the headstock geared motor (fig. 2) comes into action, to turn the double-lobed cam and thus push the notched shaft forward. This moves the pulley along the tube, and places the slide-holder above the next tank. The shaft then returns to its original position, the pawl ratcheting out of the notch in the shaft to fall into the succeeding one, ready for the next stroke. The tailstock motor is then reversed to lower the slide-holder into the tank.

Cams on the output shaft of the geared motor (fig. 2) and on the tailstock end of the slotted tube (fig. 3) actuate micro-switches to limit the travel of the rack in a vertical direction, and to start and stop the driving motors at the appropriate times.

FIG. 1 (plate). Photograph of the machine. *a*, headstock bracket; *b*, tailstock bracket.

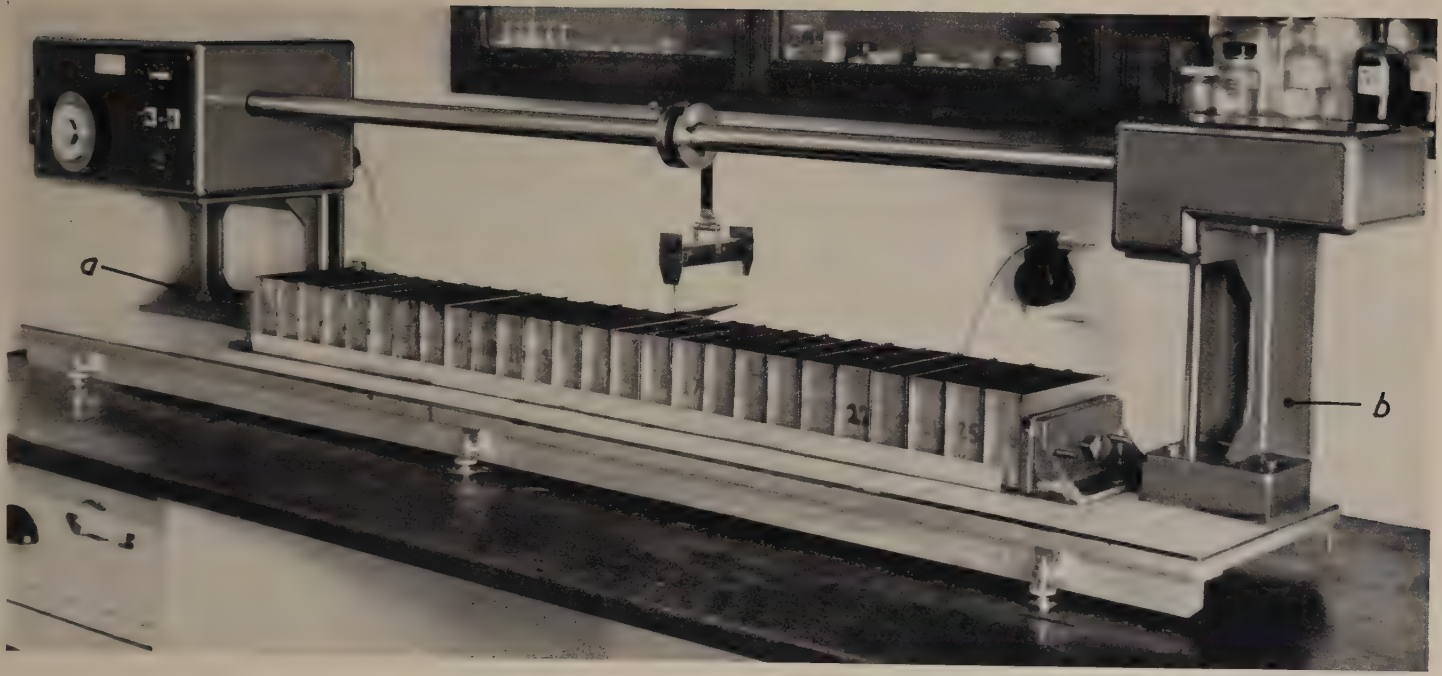

FIG. 1

S. DILWORTH

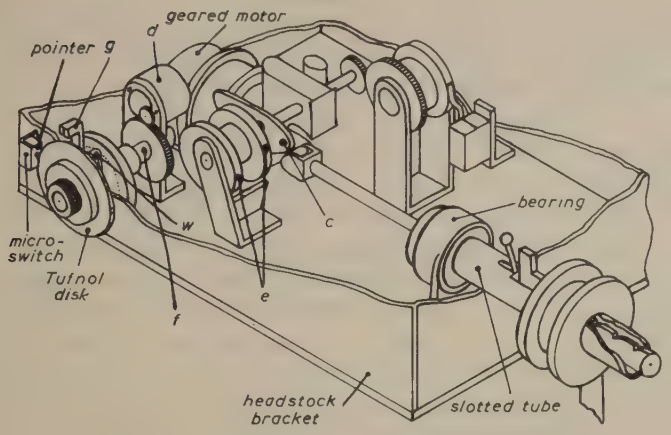

FIG. 2. Diagram of the mechanism at the headstock end. *c*, double-lobed cam; *d*, Sangamo timing motor; *e*, cams on the output shaft of the geared motor; *f*, shaft from Sangamo timing motor; *g*, indicator arm; *w*, arm engaging with countersunk holes in a disk on the shaft (*f*).

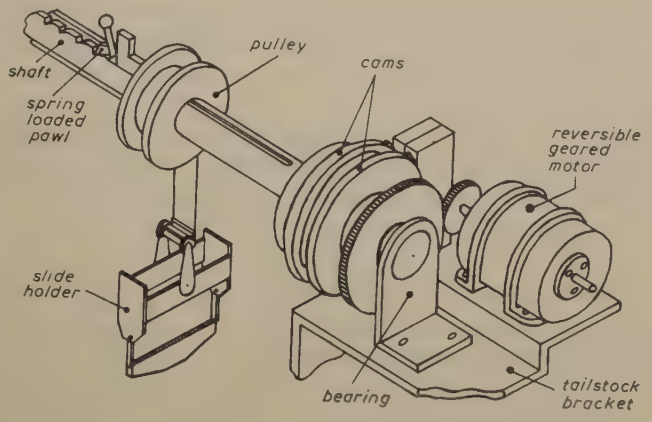

FIG. 3. Diagram of the mechanism at the tailstock end.

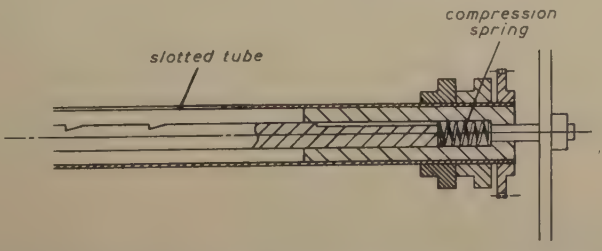

FIG. 4. Sectional view of the tailstock end of the slotted tube.

The headstock and tailstock mechanisms are completely enclosed. The starting and control switches and indicator lights are situated on a front panel of the headstock. A shaft (*f*) from the Sangamo timing motor is brought through the panel to carry the timing Tufnol disk (fig. 2).

An alarm mechanism, having an indicator arm (fig. 2, *g*) that will rotate around the edge of the disk, can be set to give either audible or visible warning at any particular tank setting. The audible signal is in the form of a buzzer inside the headstock housing, and the visual indication is a flashing light situated on the panel. Attached to the indicator arm (*g*) and situated behind the panel is another arm (fig. 2, *w*), on the end of which is a ball-catch. This catch engages a circle of countersunk holes in a disk on the shaft (fig. 2, *f*). The disk has 90 holes corresponding to 2-min periods, to cover the minimum allocation in any tank.

When the warning has been given, a switch on the headstock is then moved from 'auto' to 'manual' and an adjacent switch to 'raise'. After inspection of the slides the switch is again moved to 'lower' and the first one again reset to 'auto', when the programme will continue. At the completion of the processing, with the slides in the last tank, the warning signal is finally operated to indicate an end to the sequence. At the end of the sequence the slides remain in the last tank, which in the present system of staining contains xylene. The slides can remain in the xylene for a short time without damage to the sections, until they are removed for mounting.

My thanks are due to several colleagues at the Wool Industries Research Association in the Biology, Engineering and Electronics Departments for helpful discussions, suggestions, and technical advice, also to members of the workshop staff who constructed the machine.

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A Histochemical Study of the Connective-Tissue Sheath of the Nervous System of *Periplaneta americana*

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With one plate (fig. 1)

Summary

The connective-tissue sheath surrounding the nervous system of *Periplaneta americana* consists of two layers, the neural lamella and the sheath cells beneath it. The neural lamella is composed of a collagen-type protein and neutral muco-polysaccharide. The sheath cells possess numerous lipochondria and mitochondria; the former consist of phospholipid and some cerebroside. The cytoplasm of the sheath cells contains some RNA, glycogen, and lipid.

Introduction

THE physiological importance of the sheath surrounding the insect nervous system was first established by Hoyle (1952): it acts as a barrier to the penetration of ions and so maintains a constant ionic environment within the nervous system. This property is, perhaps, of greatest significance in herbivorous insects, where the potassium concentration in the haemolymph may be very high; but it is important in all insects.

The sheath consists of two layers, the neural lamella and the sheath cells. The neural lamella in several insects has been shown to contain a collagen-type protein and some muco-polysaccharide (Ashhurst, 1959; Baccetti, 1955, 1956, 1957; Hess, 1958 *a*, *b*; Richards and Schneider, 1958; Smith and Wigglesworth, 1959), but the sheath cells have not received so much attention. In the locust (*Locusta migratoria*), the sheath cells contain small mitochondria and large numbers of lipochondria containing cerebrosides and phospholipids (Ashhurst, 1959), but there is no information about their chemical composition in other insects. Scharrer (1939) described the storage of trypan blue granules in the sheath cells of the cockroach, but otherwise the studies of the sheath cells have been made recently with the electron microscope, by Hess (1958*b*) on the cockroach, and Edwards, Ruska, and de Harven (1958) on the wasp. The present paper describes a histochemical study of the connective-tissue sheath in *Periplaneta americana*.

The nomenclature used in this paper is that used in a previous paper (Ashhurst, 1959).

Methods

The sheath around the three thoracic ganglia was studied principally but that round the ventral nerve-cord was also examined. The ganglia and nerve-cord were removed from adult cockroaches and then treated in one of several

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different ways. They were either treated with a chemical fixative or frozen-dried, embedded in gelatin or wax, and then stained by a histological method or subjected to a histochemical test; the details of the techniques are given in the Appendix. The incubations in diastase, hyaluronidase, and saliva were carried out at 37° C; sections were incubated in diastase (0·1% in distilled water) or saliva for 1 h, but in hyaluronidase (0·1% in distilled water) for 3 h.

For chromatographic examination, the neural lamella was dissected away from the cells and nerve-fibres of 50 ganglia. The neural lamella cannot be completely freed from contamination by the sheath cells, but to reduce inaccuracies in the amino-acid analysis due to this contamination, analyses of the amino-acids of both the neural lamella and the cell fractions were made and the two were compared. The two fractions were hydrolysed in 6 N hydrochloric acid for approximately 16 h at 100° C. The hydrolysates were evaporated to dryness under reduced pressure and the residue redissolved in 10% iso-propyl alcohol. The chromatograms were two-dimensional; the first solvent being butanol / acetic acid / water (40–10–50), and the second, phenol saturated with water. The amino-acid spots were made visible by spraying with a mixture of isatin and ninhydrin in butanol (after Kolor and Roberts, 1957).

Results

The structure of the sheath

The cockroach sheath consists of two layers, the neural lamella and the sheath cells under it (fig. 1, A). The neural lamella appears to be homogeneous in the preparations used in this study. No nuclei were seen in it, in contrast to the findings of Twarog and Roeder (1956). It is between 4·5 and 10 μ thick in the region of the ganglia and between 2·5 and 6·5 μ thick around the ventral nerve-cord.

The sheath cells can be seen in some preparations to be cuboid, but the cell membranes are not always visible. The cells form a layer varying in thickness between 7 and 24 μ round the ganglia and 2 and 7 μ round the nerve-cord. The nuclei are elongated and may be 3 μ in diameter and 10 μ in length. The cytoplasm contains numerous lipochondria (fig. 1, B) between 0·5 and 4 μ in diameter and spherical mitochondria of a diameter of approximately 0·5 μ; the mitochondria can be seen in HPO preparations.

Histochemistry of the neural lamella

Carbohydrates. The neural lamella is very strongly positive to the periodic acid / Schiff (PAS) test and the reaction is not reduced in intensity by the incubation of the sections in diastase, hyaluronidase, or saliva before performing the PAS test. These results suggest that the neural lamella contains a

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Fig. 1 (plate). A, section of part of the thoracic ganglion, showing the relation of the neural lamella and sheath cells to the rest of the ganglion.

B, section of the sheath showing the lipochondria in the sheath cells. Sudan black preparation.

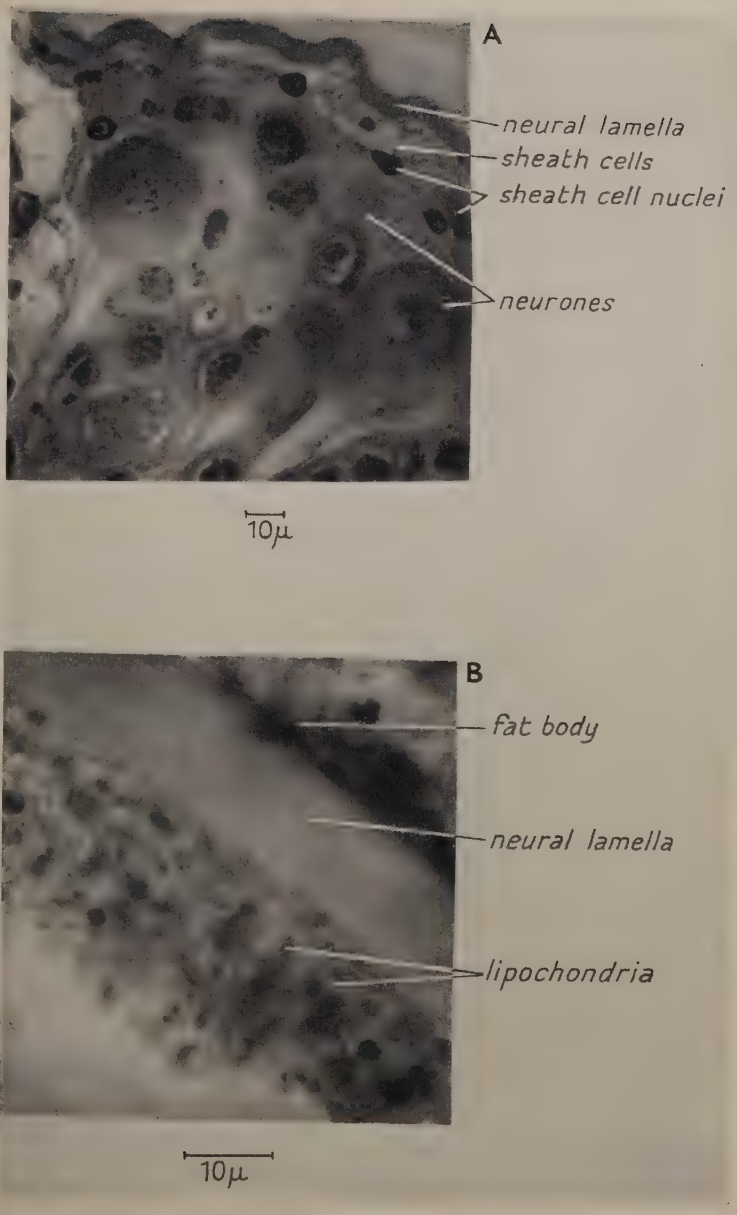

FIG. 1

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carbohydrate which is not glycogen or an acid muco-polysaccharide, since the glycogen should be removed by both diastase and saliva, and the acid muco-polysaccharides should be removed by hyaluronidase, although this is complicated by the fact that the latter are not always PAS-positive (Glegg, Eidinger, and Leblond, 1954). The neural lamella also remains PAS-positive after the benzoylation treatment; the significance of this finding will be discussed later.

The neural lamella is not chromotropic when stained with toluidine blue, but it exhibits β-metachromasia if the section is first treated with concentrated sulphuric acid, as described by Lison (1953). The reactions with toluidine blue are confirmed by the fact that the neural lamella, when subjected to the methylene blue extinction test, does not stain with methylene blue below approximately pH 5. From these results, it seems probable that the carbohydrates in the neural lamella are in the form of neutral muco-polysaccharides.

Lipids. The neural lamella appears to contain no detectable lipid, since it is negative when tested with the Sudan black and acid haematein tests. Fixation in some of the lipid unmasking fluids suggested by Bradbury and Clayton (1958) and Clayton (1959) does not reveal any 'masked' lipid.

Proteins. The neural lamella gives a positive reaction with Barnard and Danielli's (1956) test for proteins. This reaction is unaffected by preliminary benzoylation, which suggests that collagen may be present. As mentioned earlier, benzoylation does not affect the PAS reaction, which also indicates the presence of collagen (see Ashhurst, 1959). Further evidence is provided by the reactions with Baker's (1956*a*) Hg/nitrite test for tyrosine and his (1947) modification of the Sakaguchi test for arginine; the neural lamella is only very weakly positive with the former, but strongly positive with the latter.

Histochemistry of the sheath cells

Carbohydrates. The sheath cells possess a large amount of carbohydrates, since they are very strongly PAS-positive. This reaction is markedly reduced if the sections are first incubated in either saliva or diastase, which suggests that the reaction is due largely to the presence of glycogen. After Best's carmine test for glycogen, red (i.e. positive) granules, between 0·75 and 4·5 μ in diameter, are found in the cytoplasm.

Lipids. The cells are full of lipochondria, which are intensely coloured by Sudan black and Sudan IV. The cytoplasm is also coloured by these agents. The lipochondria are positive with the acid haematein test for phospholipids, which indicates that one of their component lipids is a phospholipid.

To discover whether cerebrosides are present, ganglia were extracted with hot or cold acetone. After cold acetone treatment, the lipochondria were just visible when coloured with Sudan black but much of their lipid had been displaced into the cytoplasm; but after hot acetone, the lipochondria were no longer visible and very little lipid remained in the cell. It is most probable that the reduction in total lipid by cold acetone is due to the removal of cerebrosides from the lipochondria, while the phospholipids remain; these are soluble

only in hot acetone. It is possible that some of the cytoplasmic lipid is cerebroside, but the extraction is masked by the displacement of lipid from the lipochondria by cold acetone.

Fixation in the lipid unmasking fixative suggested by Bradbury and Clayton (1958) and in mercuric chloride (Clayton, 1959) did not reveal any further lipid in the sheath cells; in fact the Sudan black coloration is less strong than after fixation in formaldehyde-calcium.

Proteins. The sheath cells are positive to all the protein tests, but it is not possible to differentiate the cell inclusions from the ground cytoplasm.

Nucleic acids. The chromatin of the sheath cells is Feulgen-positive. The cytoplasm is strongly basiphil: it is stained by the pyronin when the pyronin / methyl green test is performed on sections. This coloration is due in part to the presence of RNA; the intensity of the reaction is reduced by incubation of the sections in ribonuclease.

Alkaline phosphatase. Some frozen-dried sections were tested for alkaline phosphatase by Danielli's (1953) modification of Gomori's glycerophosphate technique. The sheath cells do not appear to possess alkaline phosphatase.

Chromatography

The amino-acids of the neural lamella were analysed as described earlier in the section on methods. The purpose of this analysis was to see if hydroxyproline, a characteristic constituent of collagen, could be identified among the amino-acids. Hydroxyproline was found to be present in hydrolysates of both the neural lamella and the sheath cells, although in the latter case 6 times as many applications of the hydrolysate were necessary to produce a definite spot.

This result gives further evidence for the presence of collagen-type protein in the neural lamella; this was inferred earlier from the reactions to the protein tests. The presence of hydroxyproline in the sheath-cell hydrolysate may be due either to small amounts to collagen in the connective tissues within the ganglion, or to contamination of the cell fraction by small amounts of the neural lamella.

Discussion

The results reported here suggest the presence of a collagen-type protein together with neutral muco-polysaccharides in the neural lamella. This agrees with the previous studies of the neural lamella of the cockroach; that is with the birefringence studies of Richards and Schneider (1958) and the electron microscope studies of Hess (1958 *a*, *b*). It must be mentioned that Richards and Schneider (1958) found evidence to suggest the presence of lipids in the neural lamella, but histochemical tests for lipids were negative. The histochemical reactions of the neural lamella of the cockroach are, therefore, the same as those of the neural lamella of the locust. But differences are apparent if it is compared with other insects which have been investigated: acid mucopolysaccharides are present in the neural lamella of *Anacridium aegyptium* (Baccetti, 1955), and the presence of lipids has been demonstrated with Sudan black in the neural lamella of lice (Pipa and Cook, 1958).

The sheath cells contain lipochondria, which appear to be identical with ıose in the locust; that is, they are composed of phospholipids and cerebro- des. The cytoplasm contains lipids and a large amount of glycogen. No other stochemical studies have been made of these cells, but Scharrer (1939) ports that the perineurium cells (sheath cells) can be distinguished from the ial cells in several ways. One difference is that if trypan blue is injected into cockroach the stain is stored by the sheath cells and none reaches the glial lls. This result may, however, in view of recent work on the function of the ıeath, be interpreted as illustrating the property of the sheath cells to act as selective ion barrier around the nervous system. In an electron-microscope udy of the sheath cells, Hess (1958*b*) describes two types of cytoplasmic in- usion, mitochondria and small vesicles and tubules with clear centres. He ncludes that the vesicles and tubules may be different manifestations in oss-section of the same inclusions. The mitochondria appear as transverse ıd longitudinal sections, the former being about 0·5 μ in diameter, the latter out 2 μ long. In the HPO preparations, the mitochondria appear spherical ıd about 0·5 μ in diameter. Furthermore, no structures bearing any resemb- nce to lipochondria are found in these electron micrographs, although the pochondria are one of the most prominent features of the Sudan prepara- ons. Their absence from the electron micrographs suggests that the dehyd- ting and embedding procedures may have removed the lipids.

A further very characteristic feature of the cockroach sheath cells is the large mount of glycogen they possess. This results in their strong PAS reaction, hich produces a sharp contrast with the ganglion cells. In the locust the ıeath cells contain less glycogen (Ashhurst, 1959). This is not easily under- andable, since it would seem that the cells in the locust must be more active ı controlling the flow of ions into the nervous system than those in the cock- ach, since the locust has much higher potassium concentrations in the aemolymph than does the cockroach. The evidence for the formation of selective ion barrier by the cells was discussed in an earlier paper.

It has been suggested (Hess, 1958 *a*, *b*; Wigglesworth, 1959 *a*, *b*) that sheath lls forming a separate layer are restricted to the ganglia, while in the ventral erve-cord and peripheral nerves they contribute to the connective tissue etween the axons. In this study, no histochemical distinction between the ıeath cells of the ganglia or nerve-cord was apparent. One important charac- ristic of the sheath cells, whether they are in the ganglia or at the extremities f the peripheral nerves, is their common function of controlling selectively ıe passage of ions into the nervous system.

I should like to thank Professor H. Graham Cannon, F.R.S., for his en- ouragement and interest while this work was in progress. I am very grateful Professor R. Dennell for his criticism of this paper. The photomicrographs ere kindly prepared by Mr. P. Howarth and Mr. R. Newton.

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APPENDIX

Table of methods and results

Test or technique	*Reference*	*Neural lamella*	*Sheath cells*
Masson	Pantin, 1948	green	red
Mallory's collagen stain	Mallory and Wright, 1924	blue	yellow
Mallory's trichrome	Mallory and Wright, 1924	blue	red
HPO for mitochondria	Baker, 1957		mitochondria black
Carbohydrates			
PAS	Pearse, 1960	+++	++
PAS with no oxidation		−	−
PAS after diastase		+++	+
PAS after hyaluronidase		+++	++
PAS after benzoylation		+	−
Toluidine blue	Baker (unpublished)	−	−
Toluidine blue after sulphuric acid	Lison, 1953	+	+
Toluidine blue after hyaluronidase		−	−
Methylene blue extinction	Pearse, 1960	about pH 5	
Best's carmine	Casselman, 1959	−	+
Lipids			
Sudan IV	Herxheimer, 1901	−	cytoplasm + lipochondria ++
Sudan black	Baker, 1945, 1949, 1956*b*	−	cytoplasm + lipochondria ++
Sudan black after cold acetone	Casselman and Baker, 1955	−	cytoplasm ++ lipochondria +
Sudan black after hot acetone	Casselman and Baker, 1955	−	cytoplasm + to − lipochondria −
Acid haematein	Baker, 1946	−	cytoplasm − lipochondria +
Acid haematein after pyridine extraction	Baker, 1946	−	cytoplasm − lipochondria − nucleus +
Nile blue	Cain, 1947	−	blue
Liebermann	Lison, 1953	−	−
Windaus	Lison, 1953	−	−
Proteins			
Coupling reaction	Barnard and Danielli, 1956	++	+
Coupling reaction after benzoylation	Barnard and Danielli, 1956	++	−
Sakaguchi	Baker, 1947	++	+
Hg/nitrite	Baker, 1956*a*	+ very weak	+
Nucleic acids			
Feulgen	Feulgen and Rossenbeck, 1924	−	nuclei ++
Feulgen control		−	−
Pyronin / methyl green	Jordan and Baker, 1955	++	++
Pyronin / methyl green after RNAase	Bradbury, 1956	++	+
Phosphastase			
Gomori's test for alkaline phosphatase	Danielli, 1953	−	−

KEY: +++ = strong reaction. ++ = medium reaction. + = weak reaction. − = no reaction.

The Connective-Tissue Sheath of the Nervous System of *Locusta migratoria*: an Electron Microscope Study

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With two plates (figs. 1 and 2)

Summary

The sheath is composed of an outer non-cellular layer, the neural lamella, and an ner layer of sheath cells. The neural lamella possesses a large number of collagen ›rils arranged in layers with differing orientations. The sheath cells are flattened on e inner surface of the lamella and the cytoplasm contains lipochondria, mitochondria, ıd small amounts of endoplasmic reticulum.

Introduction

THE connective-tissue sheath around the nervous system of insects began to attract attention after Hoyle (1953) had shown that it was essential for ıe normal functioning of the nervous system in *Locusta migratoria*. The ıeath, which consists of two layers, the neural lamella and the sheath cells, ›ntrols the passage of ions from the haemolymph into the nervous system.

In electron-microscope studies, Hess (1958) and Smith and Wigglesworth 959) have shown that collagen fibrils are present in the neural lamellae of *'eriplaneta americana* and *Rhodnius prolixus* respectively. Hess also described ıe sheath cells which are characterized by the large number of mitochondria ı the cytoplasm.

It has been suggested from histochemical evidence that the neural lamella of . *migratoria* contains collagen fibres embedded in a matrix of neutral muco- olysaccharide (Ashhurst, 1959). It was also shown that the sheath cells form layer under the neural lamella, and that the cytoplasmic inclusions of these ells are mitochondria and lipochondria. This paper describes an electron- ıicroscope study of the connective-tissue sheath in the locust. An attempt is ıade to correlate the evidence of the light-microscope study with the results btained in the electron microscope. The nomenclature of the layers of the heath is that used by Ashhurst (1959).

Methods

The locusts used in this study were all adult *L. migratoria*, supplied by the .nti-Locust Research Centre, London. Specimens were prepared for electron ıicroscopy by ultra-thin sectioning or by simple maceration.

For thin sectioning, the metathoracic ganglion and ventral nerve-cord were

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removed and immersed immediately in an osmium fixative. Two methods were used.

1. Ganglia were fixed for 1 h at 4° C in 1% osmium tetroxide in veronal-acetate buffer, pH 7·4, to which had been added calcium chloride and magnesium chloride, both to a final concentration of 0·02% (Geren and Schmitt, 1954; Palade, 1956). The material was taken directly to 70% alcohol, rinsed, and stained in a 2% solution of phosphotungstic acid in 70% alcohol for 1¼ h at room temperature.

2. Other ganglia were fixed for 2 h at 4° C in the calcium-osmium fixative (containing 1% calcium chloride) described by Chou and Meek (1958). This material was also taken from the fixative directly into 70% alcohol.

The fixed tissue was dehydrated in 90% and absolute alcohol and embedded in araldite, by the method of Glauert and Glauert (1958). Thin sections of the embedded tissue were cut on a Huxley microtome and examined in a Siemens Elmiskop I electron microscope.

Some ganglia were macerated in distilled water. One drop of the resulting suspension was placed on a carbon-filmed copper grid and dried. The grid was then shadowed with gold-palladium and examined in the electron microscope.

Results

The structure of the neural lamella

In the light microscope the neural lamella appears as a continuous, homogeneous layer surrounding the entire nervous system. Electron microscopy reveals no differentiation into regions within the layer (apart from a thin basal layer), but shows that the lamella contains numerous fibrils in varying orientations (fig. 1, A, B). Occasionally remnants of the fat-body, containing tracheae, can be seen adhering to the outer surface; in life the ventral nerve-cord and ganglia are surrounded completely by the fat-body. In some instances tracheae can be observed penetrating the neural lamella.

At higher magnifications of thin sections of the neural lamella the fibrils can be seen to be banded. Longitudinally sectioned fibrils of material treated with phosphotungstic acid possess very obvious banding, with a periodicity of 400 to 500 Å and up to 6 inter-bands per period (fig. 2, A, B). Banding is also observed in many of the fibrils occurring in the metal-shadowed debris of macerated ganglia (fig. 2, C). These fibrils, which almost certainly come from

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Fig. 1 (plate). A, low-magnification electron micrograph of the connective-tissue sheath surrounding the metathoracic ganglion of *L. migratoria*, showing the outer, fibrillar neural lamella (*nl*) and the inner layer of sheath cells (*sc*). The sheath cells are irregular in shape and the numerous cytoplasmic membranes (such as those marked *cm*) probably mark the limits of individual cells. Numerous lipochondria (*l*) and mitochondria (*m*) are present in the cytoplasm; the endoplasmic reticulum is not clearly resolved at this magnification. An amorphous basal layer (*b*), shown more clearly in fig. 2, D, lies between the neural lamella and the sheath cells. The innermost limit (*i*) of the sheath cells exhibits cytoplasm of high electron density.

B, the neural lamella, showing fibrils in varying orientations. The longitudinally sectioned fibrils, in layers approximately parallel to the outer surface of the lamella, show a banded structure with a periodicity of about 450 Å (arrows). Obliquely oriented fibrils occur between these layers.

FIG. 1

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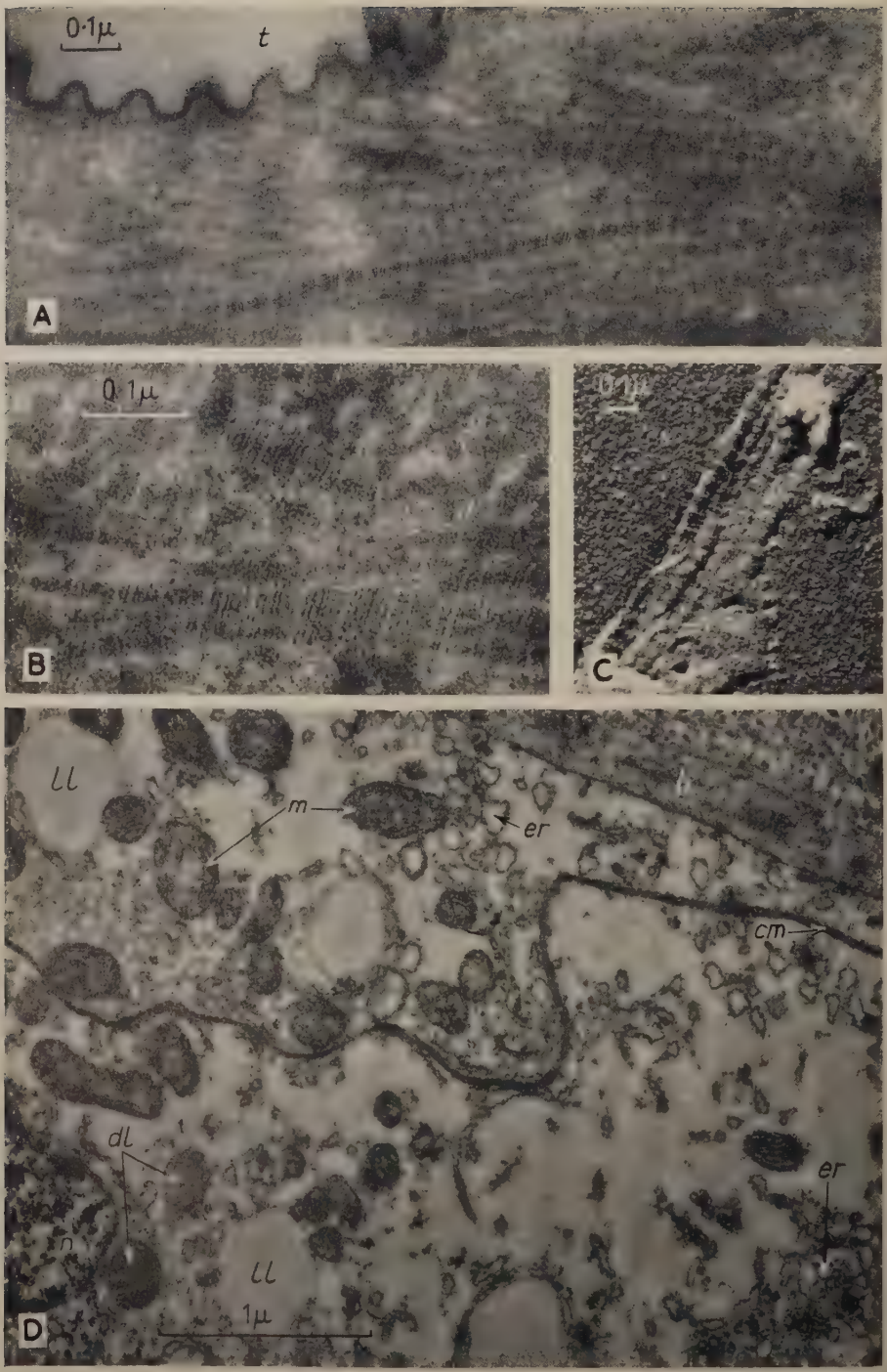

FIG. 2

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ıe neural lamella, show a periodicity of between 600 and 650 Å. In addition few smaller, non-banded fibrils (diameter 200 Å) are found in the macerated anglia.

The nature of the banding and the periodicity both suggest that the banded brils are collagen. The range in the periodicity of the banding comes within ıe limits normally observed in vertebrate collagens prepared by similar chniques. The smaller periodicity in the sectioned fibrils is to be expected in iew of the shrinkage caused by fixation, dehydration, and cutting.

In transverse section the fibrils show diffuse, irregular outlines (as observed y Gray, 1959) in sharp contrast to the characteristic circular outlines of verte- rate collagens (fig. 2, B).

The spatial arrangement of the fibrils in the lamella is not readily deduced om isolated thin sections but the results indicate that the orientation of the brils is not entirely random. The longest (i.e. longitudinally sectioned) brils, occurring singly and in groups, are usually arranged approximately arallel to the outer surface of the lamella. Fibrils lying at an angle to the outer ırface are invariably very short. Moreover, layers of the longest fibrils, in the arallel orientation, tend to alternate with layers of the shorter, obliquely riented fibrils. In some instances this repeating pattern is manifest as a ıerring-bone' arrangement of fibrils (as in fig. 1, B). In other instances, pre- ımably when the plane of sectioning is roughly normal to the surface of the mella, layers of longitudinally sectioned fibrils alternate with thicker layers of brils cut in approximately transverse section (as seen at low magnification in g. 1, A). These observations suggest that the fibrils lie in layers approximately arallel to the outer surface of the lamella but with the fibril direction changing om one layer to another.

Between the fibrillar neural lamella and the underlying sheath cells there ccurs an amorphous basal layer, 0·2 to 0·3 μ in thickness (figs. 1, A; 2, D). his layer was not apparent in the light microscope; its composition and ınction are unknown.

he structure of the sheath cells

The sheath cells form a continuous layer underlying the neural lamella ig. 1, A). The cells are very irregular in shape; in the light microscope it is npossible to determine the limits of these cells. In electron micrographs, owever, a series of membranes can be seen crossing the cytoplasm. It is

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FIG. 2 (plate). A, electron micrograph of part of the neural lamella of *L. migratoria* with anded fibrils and a trachea (*t*) in close proximity.

B, longitudinal sections of banded fibrils in the neural lamella at high magnification, showing ıe inter-band structure. The transverse sections of fibrils show that they are non-circular in oss-section.

C, banded fibrils from macerated ganglia, shadowed with gold-palladium. The periodicity of ıe banding is about 600 Å. (Electron micrograph taken by Dr. R. Peach.)

D, parts of two sheath cells showing the double bounding membrane (*cm*) between the cells ıd various cytoplasmic inclusions (*m*, mitochondria; *ll*, 'light' lipochondria; *dl*, 'dark' lipo- ıondria; *er*, endoplasmic reticulum; *n*, nucleus). The amorphous basal layer (*b*) is also seen, eparating the neural lamella from the sheath cells.

suggested that these membranes are the cell membranes of adjacent sheat cells, the irregularities in the shape of the cells being responsible for the manne in which these membranes ramify within this cell-layer. The innermost par of the cells is characterized in some preparations by a region of very electron dense cytoplasm, containing many cell inclusions. It is possible that this i either a fixation artifact or is due to a modification of the cytoplasm, correlate with the physiological function of these cells, that is, with their properties c controlling the passage of ions into the nervous system (Hoyle, 1953; Twaro and Roeder, 1956).

The nuclei are elongated and are surrounded by a continuous double mem brane. Clumps of chromatin occur inside. There are three main types of cyto plasmic inclusions (fig. 2, D). The most obvious of these are the mitochondria which are very numerous. Their shape in sections is variable, but they are al surrounded by a double membrane, with an irregular series of double mem branes forming the cristae inside. Another type of inclusion is a rounded homogeneous body. These can be divided into two groups, large and small the larger ones are less electron dense than the small ones. These cell inclusion are identified as the lipochondria, which are a very obvious feature of Sudan black preparations. In addition to the cell inclusions mentioned already numerous small vesicles are scattered throughout the cytoplasm. This system of vesicles and granules forms the endoplasmic reticulum of these cells.

Discussion

The results of the electron-microscope study reported in this paper confirm the conclusions drawn from the histochemical study of the connective-tissue sheath in the locust (Ashhurst, 1959). It was suggested that the neural lamella is composed of a collagen-type protein in a matrix of neutral mucopoly saccharides. Fibrils exhibiting a periodicity characteristic of collagen are found in abundance in the neural lamella. The mucopolysaccharide probably con tributes to the debris around these collagen fibrils. Similar fibrils have been described in *R. prolixus* and *P. americana* (Smith and Wigglesworth, 1958; Hess, 1958). The arrangement of the fibrils in *Locusta* is similar to that in *Rhodnius*; there are several layers of fibrils, each layer having a different orientation to the surface of the neural lamella. But there is no distinct outer layer as described by Hess in *Periplaneta*, nor are the layers of fibrils limited to three. It has been suggested by Gray (1959) that the presence of collagen fibrils in insects may preclude the presence of tracheae; he observed that collagen fibrils were present in the intraganglionic connective tissue of the auditory ganglion (Müller's organ) of *L. migratoria*, but that tracheae were absent. The work described here shows, however, that collagen fibrils and tracheae may occur in close proximity in the neural lamella surrounding the metathoracic ganglion (see, for example, fig. 2, A). This suggests that the auditory ganglion is a special case and that the presence of collagen fibrils is not dependent on the absence of a supporting tracheal system.

The cytoplasmic inclusions seen in the sheath cells in the light microscope

can be identified in the electron micrographs. The only discrepancy is the apparent scarcity of the lipochondria, compared to the large numbers seen in Sudan black preparations. This may be due in part to the dehydration necessary in the preparation of the tissues for electron microscopy, for the tissues coloured with Sudan black were not dehydrated. The only other description of the sheath cells in insects is given by Hess (1958); he describes the inclusions of the sheath cells of *Periplaneta* as mitochondria and small vesicles. He finds no lipochondria, but these are a prominent feature of Sudan black preparations of the cells (Ashhurst, in press). It is obvious that some lipid is removed by the preparative techniques involved in electron microscopy.

It is known that the connective-tissue sheath controls the passage of ions from the haemolymph to the interior of the ganglion. Some insects have very high concentrations of potassium ions in the haemolymph, and if this concentration were present around the axons, conduction in the nerves would be blocked (Hoyle, 1953). The sheath cells appear to form the actual barrier to the ions (Hoyle, 1953; Twarog and Roeder, 1956). No evidence has been found which would disprove this idea. Edwards, Ruska, and de Harven (1958), after studying the fine structure of the peripheral nerves in the wasp, went further in suggesting that while the sheath cells provide a selective ion barrier, the neural lamella controls the rate of flow of ions to the sheath cells.

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Tyrosinase Activity in the Pigmented Cells of the Nucleus Substantiae Nigrae

II. Further Observations on Monophenolase Activity

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With one plate (fig. 1)

Summary

In a previous investigation of tyrosinase activity in the cells of the nucleus substantiae nigrae of adult cats and monkeys, diphenolase, but not monophenolase activity, was detected. This is possibly due to inhibition of monophenolase. When the copper adicle in tyrosinase is oxidized, monophenolase cannot be detected, but reducing gents activate and unmask this component of tyrosinase.

The effects of reducing agents in activating monophenolase have been investigated. Tyrosine is converted to melanin by monophenolase in adult nigra cells of the cat and nonkey when the enzyme is activated by the reducing agents L-dopa, L-ascorbic acid, nd L-adrenaline. Variations in physical factors influence the intensity of the activated nonophenolase reaction resulting from incubation of sections in tyrosine-dopa nixtures; the reaction is inhibited by general enzyme inhibitors and specific tyrosinase nhibitors.

The significance of the results in relation to tyrosinase activity in adult nigra cells is iscussed.

Introduction

THE pigmented cells of the nucleus substantiae nigrae of adult cats and monkeys convert 3,4-dihydroxyphenylalanine (dopa) to melanin pigment *in vitro*, and evidence has been presented to show that this is due to the presence of the diphenolase activity of the enzyme tyrosinase in the cells (Marsden, 1960). Monophenolase activity of tyrosinase, however, cannot be detected and adult nigra cells are apparently incapable of oxidizing tyrosine to dopa, which is the first step in melanin formation. In order to show that the pigment in nigra cells is formed by the action of tyrosinase on the amino acid tyrosine, the metabolic system responsible for the production of melanin in other pigmented tissues in the mammalian body (Fitzpatrick, Brunet, and Kukita, 1958; Fitzpatrick and Kukita, 1959), it is necessary to demonstrate the presence not only of diphenolase activity, but also of the monophenolase activity of tyrosinase.

The oxidation of tyrosine to dopa by monophenolase activity is slow in onset, increasing in rapidity after an initial time-lag or induction period (Lerner and Fitzpatrick, 1950). The duration of the induction period is dependent on the redox potential of the system; reducing agents shorten the period, by lowering the redox potential, while conversely, oxidizing agents increase the time lag (Figge, 1948). No similar induction period exists in the

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oxidation of dopa to dopa-quinone by diphenolase activity. The oxygen trace studies of Mason, Fowlks, and Peterson (1955) have shed new light on the mechanism of tyrosinase action and the method whereby reducing agents shorten the induction period in tyrosine oxidation. Mason (1956) has suggested that the copper radicle of tyrosinase may exist in either the reduced or oxidized state. In life, tyrosinase may exist as a reduced enzyme active towards both tyrosine and dopa, or, because of a high prevailing redox potential, as an oxidized form, which is inert but capable of activation towards tyrosine, while remaining readily active towards dopa. In the presence of reducing agents two cupric atoms in tyrosinase are reduced to cuprous atoms, and the enzyme becomes active towards tyrosine. This mechanism may be invoked to explain the apparent absence of monophenolase activity in adult pigmented nigral cells; the tyrosinase therein perhaps being in the oxidized state.

The most effective agent for shortening the induction period in tyrosine oxidation is dopa, which, in addition to serving as a substrate, can itself function as an activator by reducing cupric tyrosinase to cuprous tyrosinase with the consequent unmasking of monophenolase activity (Lerner, Fitzpatrick, Calkins, and Summerson, 1949). Tyrosine oxidation by tyrosinase is therefore an autocatalytic process. Ascorbic acid (Arman and Jones, 1949) and substances structurally related to dopa, such as adrenaline (Lerner, Fitzpatrick, and Summerson, 1949) also shorten the induction period by their reducing action, but not nearly so effectively as dopa. The possibility that reducing agents might unmask monophenolase activity has been investigated in the present study.

Materials and Methods

As in the first part of these investigations, frozen sections were obtained from the mid-brain of adult cats and monkeys, previously fixed for 3 h in 10% neutral formalin (Marsden, 1960). Sections were incubated for 24 to 48 h at 37° C in a solution containing 1 : 1000 L-tyrosine in 0·1 M phosphate buffer at pH 6·8, to which were added potential activators of monophenolase activity. The reducing agents, L-dopa, L-ascorbic acid, L-adrenaline, and sodium hydrosulphite were separately added to the incubating solution at concentrations of 0·005%. Sections incubated in L-tyrosine alone or in the same concentrations of the individual reducing agents alone were used as controls. Sections were also incubated in buffer solution.

The addition of L-dopa to the incubating solution of L-tyrosine was most likely to activate monophenolase activity, if tyrosinase was present in the oxidized state in adult nigra cells. The existence of monophenolase activity could only be demonstrated by showing that the tyrosine was converted to melanin. It was, of course, possible that, in a tyrosine-dopa mixture, the dopa was acting as a substrate for tyrosinase, resulting in blackening of nigra cells by the melanin produced by diphenolase, rather than by monophenolase activity. To eliminate this possibility, sections were incubated in radioactive L-tyrosine C^{14}, to which non-radioactive L-dopa was added, and were subsequently

subjected to autoradiography, according to the method of Kukita and Fitzpatrick (1955). Control sections were incubated in this solution with the addition of 0·01 M sodium diethyldithiocarbamate, a copper-binding inhibitor of tyrosinase; sections were also incubated in L-tyrosine C^{14} alone.

Experiments were conducted to determine the nature of the reaction resulting from incubation of sections in tyrosine-dopa mixtures. The effects of variations of physical factors on the reaction were investigated and inhibitors of melanin formation were employed. Sections were incubated in buffered L-tyrosine, to which L-dopa was added, and the pH concentration of L-tyrosine, the duration of incubation, and the temperature were independently varied as in the previous investigation (Marsden, 1960). Sections were incubated for 24 h at 37° C in 1 : 1000 L-tyrosine in phosphate buffer at pH 6·8, to which the following inhibitors of melanin formation were added separately: hydrogen sulphide, sulphur dioxide, potassium cyanide, sodium azide, phenylurethrane, 'tween 20', hydroquinone, sodium diethyldithiocarbamate, thiourea, phenylthiourea, α-naphthylthiourea, and 4-chlororesorcinol. The modes of action of these inhibitors and the concentrations employed were discussed in the previous investigation (Marsden, 1960).

Results

After incubation of mid-brain sections of adult cat and monkey in L-tyrosine, to which L-dopa had been added, the cells of the nucleus substantiae nigrae were blackened (fig. 1, A). No such blackening occurred in control sections (fig. 1, B). This blackening resembled that observed after incubation of sections in L-dopa alone, and was due to the presence of many granules of black melanin pigment in the cytoplasm of the nigra cells. Although a number of isolated cells in the reticular formation of the mid-brain were also blackened, the cells of other nuclear groups were free of pigment. Incubation of sections in L-tyrosine, to which ascorbic acid (0·005%) or L-adrenaline (0·005%) had been added, produced a similar result, although the blackening of nigra cells was less intense than when dopa was used as the activator (fig. 1, C). No blackening of nigra cells followed incubation of sections in L-tyrosine with sodium hydrosulphite (0·005%). In sections incubated in L-dopa (0·005%) or L-adrenaline (0·005%) alone the nigra cells were darker than in control sections incubated in buffer alone, but the intensity of the reaction was slight, and the number of darkened cells was smaller than in sections incubated in tyrosine-dopa or tyrosine-adrenaline mixtures. No change could be detected between sections incubated in L-ascorbic (0·005%) or sodium hydrosulphite (0·005%) alone and similar material incubated in buffer solution alone.

In the autoradiographs, concentrations of silver grains were deposited over the blackened nigra cells (fig. 1, D, E); but no such deposition of silver occurred in other cells in the same section, or in the control sections (fig. 1, F).

The variations of pH, concentration of L-tyrosine, duration of incubation, and temperature all affected the intensity of blackening of nigra cells which resulted from incubation of sections in tyrosine-dopa mixtures. The reaction

occurred only between pH 6·8 and 7·4; its intensity increased with greater concentrations of L-tyrosine or with increased periods of incubation; no reaction took place at 4° C, or at 60° C, but specific blackening occurred after 72 h of incubation at 18° C, or 24 h of incubation at 37° C. The blackening of nigra cells resulting from incubation of sections in tyrosine-dopa mixtures was completely suppressed by the majority of inhibitors employed, namely hydrogen sulphide, sulphur dioxide, potassium cyanide, 'tween 20', hydroquinone, sodium diethyldithiocarbamate, thiourea, phenylthiourea, α-naphthylthiourea, and 4-chlororesorcinol, but sodium azide and phenylurethrane did not prevent the reaction.

Discussion

The monophenolase activity of tyrosinase could not be detected in the adult nigra cells of the cat and monkey, although diphenolase activity was present (Marsden, 1960). Dopa, but not tyrosine, was converted to melanin by nigra cells. However, it has been found possible to promote the conversion of tyrosine to melanin in nigra cells by the use of reducing agents in the reaction. Blackening of nigra cells took place when mid-brain sections were incubated in L-tyrosine, to which reducing agents such as L-dopa, L-ascorbic acid, or L-adrenaline were added. The apparent failure of sodium hydrosulphite in this respect is inexplicable. By far the most effective of these agents was L-dopa, which might itself have acted as a substrate for the diphenolase activity of tyrosinase. The existence of monophenolase activity in nigra cells could only be demonstrated by showing that tyrosine was converted to melanin therein.

Certain evidence adduced from the experiments conducted in this study confirmed that the nigra cells possessed the capacity to promote the conversion of tyrosine to melanin, in the presence of reducing agents. The deposition of silver in autoradiographs prepared after incubation of sections in radioactive L-tyrosine C^{14}, to which non-radioactive dopa had been added, must have been due to the uptake of the labelled amino-acid. Tyrosine is water-soluble, and, during processing, the sections were subjected to a period of 8 h of washing to remove all free tyrosine. The silver deposition was observed over the nigra cells, which were blackened with melanin; this indicated the presence of radioactivity in them. The radioactivity could only have been due to the

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Fig. 1 (plate). A, the nucleus substantiae nigrae of the adult monkey after incubation in L-tyrosine, with the addition of L-dopa.

B, the nucleus substantiae nigrae of the adult monkey after incubation in L-tyrosine alone.

C, the nucleus substantiae nigrae of the adult monkey after incubation in L-tyrosine, with the addition of L-ascorbic acid.

D, a cell of the nucleus substantiae nigrae of the adult monkey after incubation in radioactive L-tyrosine C^{14}, with the addition of L-dopa. Cell in focus.

E, the same cell as in D. The overlying photographic emulsion in focus. Silver granules are concentrated over the nigra cell.

F, the nucleus substantiae nigrae of the adult monkey after incubation in radioactive L-tyrosine C^{14} alone. No silver grains are visible over the nigra cells. Haematoxylin and eosine.

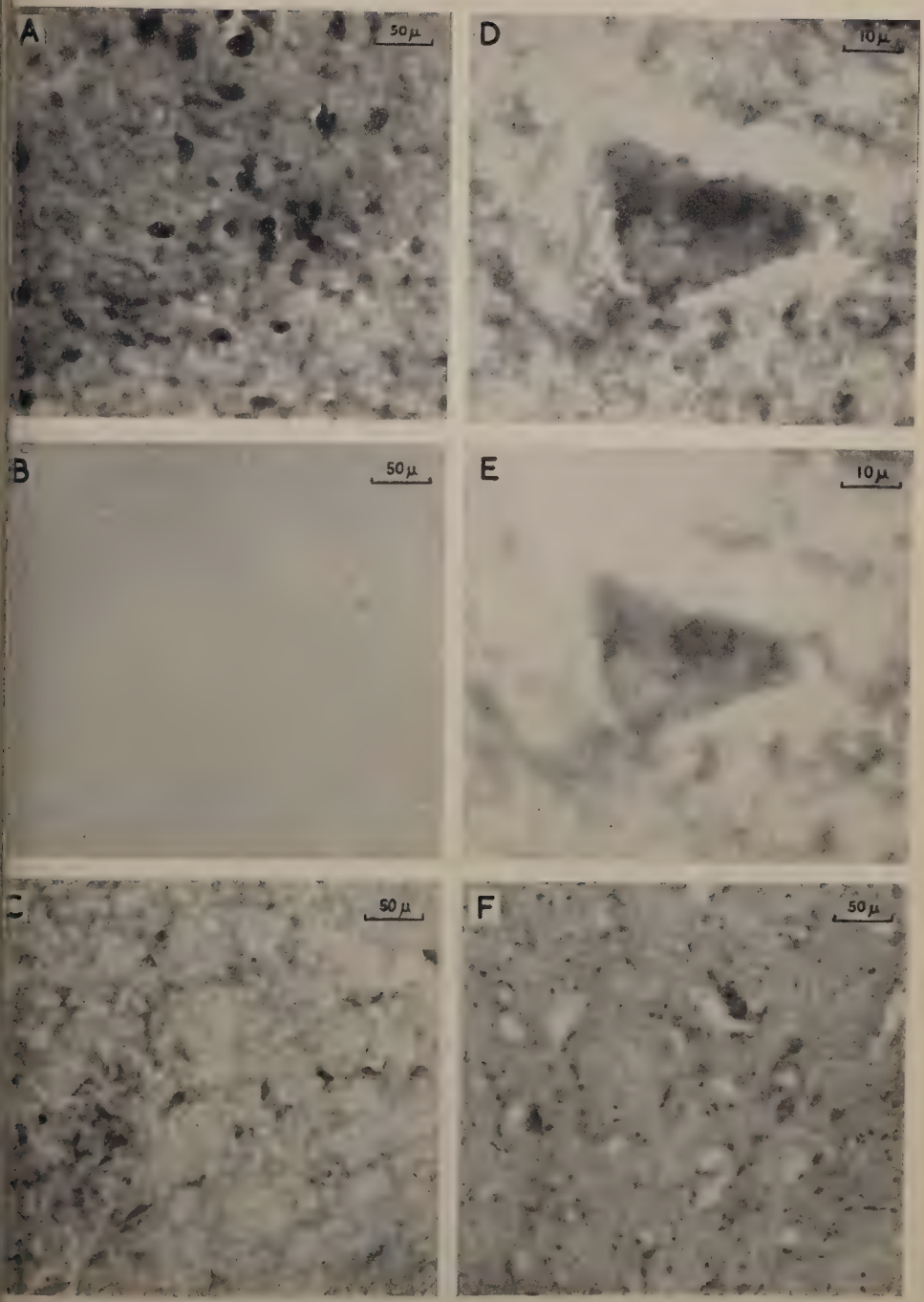

FIG. 1

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conversion of the labelled L-tyrosine to melanin, which, being insoluble in water, remained after washing. The absence of radioactivity in control sections indicated that the period of washing was effective in removing all free L-tyrosine C^{14}. In addition, the inhibitors 'tween 20' and hydroquinone prevented blackening of nigra cells incubated in tyrosine and dopa, but did not interfere with blackening of nigra cells when incubated in dopa alone (Marsden, 1960). These two substances have been shown to prevent the conversion of tyrosine to dopa, but not the subsequent oxidation of dopa to melanin (Lerner and Fitzpatrick, 1950; Denton, Lerner, and Fitzpatrick, 1952).

These findings indicated that tyrosine was converted to melanin in the nigra cells of the adult cat and monkey, when reducing agents were present. The factor responsible for this reaction was probably the monophenolase component of tyrosinase, and the actions of the remaining inhibitors confirmed this conclusion. The general enzyme inhibitors—hydrogen sulphide, sulphur dioxide, and potassium cyanide—prevented the reaction. The cytochrome oxidase inhibitors, sodium azide, and phenylurethrane (Pearse, 1953), did not prevent blackening of nigra cells after incubation in tyrosine and dopa, and this indicated that this group of enzymes was not responsible for the reaction. However, specific inhibitors of tyrosinase prevented the conversion of tyrosine to melanin by nigra cells. The modes of action of these inhibitors were discussed in the previous investigation (Marsden, 1960). These results suggested that tyrosinase was present in the oxidized state in adult nigra cells, and hence required activation by reducing agents to demonstrate its monophenolase activity.

Recently Shimizu, Matsunami, and Onishi (1960) demonstrated high concentrations of ascorbic acid in the nucleus locus coeruleus, the dorsal motor nucleus of the vagus, and the area postrema. Pigment similar to that found in the nucleus substantiae nigrae is present in these sites (Olszewski and Baxter, 1954) and may reasonably be presumed to be formed by the action of tyrosinase on tyrosine. The presence of ascorbic acid in these particular nerve-cells provides an activator of monophenolase activity, and thus pigment formation from tyrosine could take place at these sites.

No function can be ascribed to pigment in the cells of the nucleus substantiae nigrae or in other nerve-cells. It is possible that melanin in the brain is a by-product of some other essential metabolic process occurring in pigmented cells. Tyrosine is the precursor, not only of melanin, but also of the catechol amine dopamine, which has been demonstrated in the brains of man and animals, especially in the basal ganglia (Carlsson, Lindqvist, Magnusson, and Waldeck, 1958; Bertler and Rosengren, 1959*a*), and, ultimately of noradrenaline and adrenaline (Lerner, 1953), which have also been shown to be present in nervous tissue (Vogt, 1954). Bertler and Rosengren (1959*b*) have concluded that dopamine has a specific function in the brain, concerned with motor activity, while Vogt (1954) suggested that noradrenaline and adrenaline are concerned with sympathetic activities in the brain. Recently Bertler (1960) has

shown the presence of large amounts of dopamine in the human substantia nigra, a nucleus recognized as an integral part of the extrapyramidal motor system. The first step in the synthesis of dopamine, noradrenaline, or adrenaline is the oxidation of tyrosine to dopa, and the present investigation has demonstrated the presence of the enzyme responsible for this reaction, tyrosinase, in the pigmented cells of the nucleus substantiae nigrae of the adult cat and monkey.

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Secretory Structures associated with the Neurosecretory System of the Immature Scorpion, *Heterometrus swammerdami*

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With one plate (fig. 2)

Summary

Certain previously unknown structures, probably endocrine in nature, are described. The blind 'end-organ' which is present and shows signs of activity in the pre-moult stage shows no noticeable sign of activity in the post-moult stage. In certain respects it is comparable to the 'anterior organ' of spiders, which has been homologized with the prothoracic glands of insects.

In the rostral region of the scorpion, where the rostral nerve ramifies, an accumulation of leucocytes is found; these show signs of secretory activity. Both at the origin of the rostral nerve and also where it ramifies, neurosecretory material is seen. This rostral structure is comparable in certain aspects with the rostral organ of spiders. Leucocytes occur not only in the rostral organ but also in association with the 'end-organ'.

The presence of two ganglionic masses, above and below the stomodaeal commissure, suggests the possibility of the sympathetic (stomatogastric) ganglion of the scorpion being a composite structure consisting of the frontal ganglion, the corpus cardiacum, and possibly the hypocerebral ganglion. A hypocerebral ganglion is absent in the adult.

Introduction

VERY little is known about the endocrinology of arachnids, though some work has been done on their neurosecretory organs (Gabe, 1955). During the course of a detailed study of the neurosecretory system of several arachnids, certain hitherto unknown structures of a secretory nature have been observed in young scorpions. A description of the structures is presented here, and a comparison is made with structures already known in other arachnids and in insects.

Material and Methods

Different developmental stages of *Heterometrus swammerdami*, including the pre- and post-moult stages of the young forms, were studied. The entire prosoma of the young animal was fixed in Bouin's fluid made up with a Ringer solution, adapted for use with scorpions, in place of water. Before fixation the pedipalps and the legs were cut off as near the base as possible to facilitate thorough penetration of the fixative. The material was embedded in paraffin after clearing in methyl benzoate containing 0·5% of collodion. Serial sections were cut at 6 to 8 μ and stained with Gomori's (1941) chrome alum haematoxylin/phloxin or Mallory's triple stain.

[Quarterly Journal of Microscopical Science, Vol. 102, part 4, pp. 475–479, 1961.]

Observations

In young stages of *H. swammerdami* (but not in the adult) the supra-oesophageal ganglion is situated far behind the anterior region of the sub-oesophageal nerve-mass. From the pedipalpal ganglion a nerve arises dorsally and laterally, to end blindly in a small bulb-like end-organ, without reaching

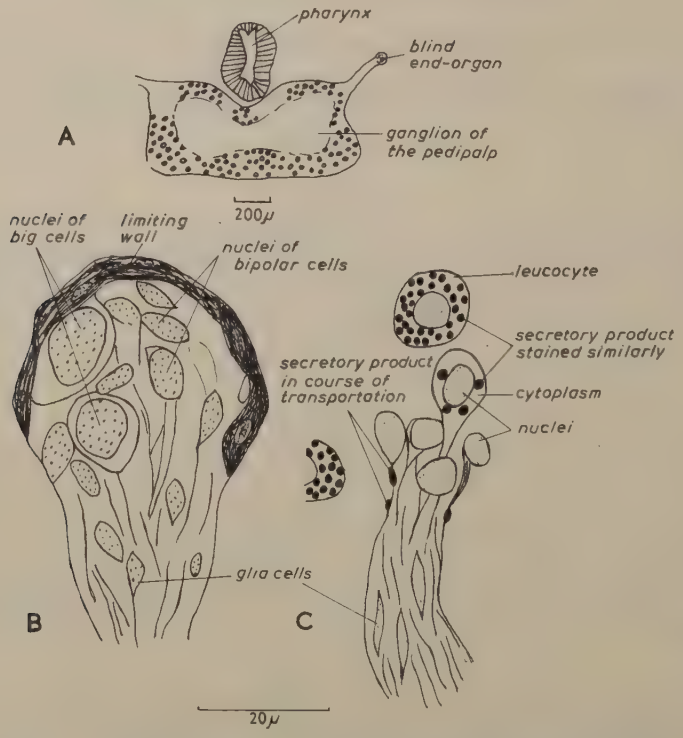

Fig. 1. A, anterior region of the sub-oesophageal ganglion in transverse section, to show the location of the blind end-organ. B, the blind end-organ in post-moult stage. C, the blind end-organ in the pre-moult stage. (Gomori's chrome alum haematoxylin/phloxin.)

the exterior. The end-organ is about 40 μ across at its broadest part. This structure is paired and arranged symmetrically on the two sides of the body (fig. 1, A).

When examined under medium power or oil immersion, this tiny organ reveals two types of cells, the processes of which enter the nerve. A few big cells with a very large nucleus constitute the first type. The cells of the other type are more numerous, much smaller, and bipolar. The cytoplasm of both types of cell is feebly stained with chromic haematoxylin and with the aniline blue of Mallory's stain. Nucleoli are not apparent. The chromatin is in the form of big beads. There are large intercellular spaces. The entire organ is enveloped by a wall one cell thick. The nuclei of these cells are also clearly

seen in the post-moult stage (fig. 1, B). The whole organ, limited by a wall one cell thick, is bathed in the haemocoelic fluid. Leucocytes occur in the vicinity of the organ. Wigglesworth (1956) demonstrated the endocrine role of amoebocytes in the moulting of *Rhodnius*. In the pre-moult stage the limiting wall of the blind end-organ is absent and the leucocytes are very near the cells of the organ. The secretory products of the leucocytes are stained with acid fuchsin, and with chromic haematoxylin after oxidation. Similar products are found in the cytoplasm of some of the cells of the organ in the pre-moult stage. The product can also be seen in the course of transportation along the extension of the cytoplasm into a process that enters the nerve. These indications of active secretion were not found in the adult.

An accumulation of leucocytes occurs in the rostrum (fig. 2, D). The secretory (possibly endocrine) nature of these cells can be inferred from their staining reactions. The secretory product takes up acid fuchsin, and chromic haematoxylin after oxidation. The secretory product is so abundant at times that it occupies almost the entire cytoplasm. The rostral nerve branches off at this region of the accumulation of cells. The cells are similar to those found in association with the blind end-organ.

Two ganglionic cell-masses are situated near the stomodaeal commissure (fig. 2, E). Just above this commissure there is a small, unpaired mass of ganglionic cells. Immediately below the stomodaeal commissure but above the stomodaeum and attached to it, there is another group of ganglionic cells. There is an axonal connexion between the two ganglionic masses. Axons from the hypostomodaeal commissural ganglionic mass run to the suprastomodaeal commissural ganglionic cells. From this mass some fibres are given out into the stomodaeal commissure.

Discussion

A comparison of the blind end-organs of the pre- and post-moult stages suggests that it plays a role in the moulting process. The organ differs markedly in some respects from the 'anterior organ' of spiders (Le Gendre, 1958), which is homologized with the prothoracic glands of insects. The anterior organ of spiders is unpaired, devoid of a limiting wall or envelope, and supplied with two unpaired nerves arising from the ventral surface of the pedipalpal ganglion. The blind end-organ of the scorpion has a limiting wall in the post-moult stage. It is paired and symmetrical and the nerve supply is from the dorsal and lateral parts of the pedipalpal ganglion. Both the structures are bathed in the haemocoelic fluid.

The rostral organ of spiders (Le Gendre, 1958) differs markedly from the rostral accumulation of leucocytes in the scorpion. The rostral organ of spiders is supplied by the unpaired rostral root and is formed of 3 or 4 large ganglionic cells, forming a part of the frontal ganglion. In the scorpion the rostral nerve branches off at the site of the accumulation of leucocytes. The rostral nerve itself is formed at least in part from the axons of the neurosecretory cells. This suggests the possibility that the neurosecretory product is elaborated by the

leucocytes accumulated here after having been transported along the rostral nerve. It was observed that both at the base of the rostral nerve and at the point where the nerve ramifies, a neurosecretory product is present.

The stomodaeal commissure is thought to be formed from the frontal ganglion—the anterior face of the cheliceran ganglion—and the paired rostral roots. If the suprastomodaeal commissural ganglion is compared with the frontal ganglion (because it is situated above and anterior to the stomodaeal commissure and gives off axons to both the commissure and the rostral nerve), it will equally be possible to compare the hypostomodaeal commissural ganglion to the 'II organ' of Schneider in spiders. This structure is formed from the dorsal wall of the stomodaeum, and remains very close to the digestive tube (in Agelenidae). Whereas it is a paired structure in spiders, it is unpaired in the scorpion. An homology has been drawn by Le Gendre between the 'II organ' and the corpus cardiacum of insects, and between the stomatogastric ganglion of scorpions and the corpus cardiacum of insects by Gabe (1955).

If the frontal ganglion of the insect were shifted posteriorly so as to touch the anterior limit of the brain, and if the hypocerebral ganglion along with the corpus cardiacum moved forward to unite with the frontal ganglion, there would be a resemblance to the stomatogastric system of the young scorpion. The observations from our own preparations indicate that the axons of the protocerebral neurosecretory cells end in this region. The accumulation of neurosecretory products is clearly seen. These facts suggest that the stomatogastric ganglion of scorpions should be homologized with the corpus cardiacum of insects. In scorpions there would be no need for a recurrent nerve, and it is indeed absent at all stages. An axonic connexion is, however, present in young scorpions between the two ganglionic masses (the frontal and 'hypocerebral' ganglia). The hypocerebral ganglion is either completely fused with the corpus cardiacum or disappears in the adult. This suggests that the stomatogastric ganglion of scorpion is a composite structure formed of the frontal ganglion and the corpus cardiacum, and possibly also of the hypocerebral ganglion

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FIG. 2 (plate). A, transverse section passing through the anterior region of the cephalothorax of a post-moult individual. Note the blind end-organ, visible dorsolaterally on the right side. The scale represents 188 μ. (Gomori's chrome alum haematoxylin/phloxin.)

B, the blind end-organ in the post-moult individual. Note two types of cells in the organ, the cellular limiting wall, and 4 leucocytes adhering to the stalk of the organ. The scale represents 25 μ. (Staining as in A).

C, the blind end-organ in the pre-moult individual. Two leucocytes are seen very close to the cells of the blind end-organ, which is without a limiting wall. The scale represents 18 μ. (Staining as in A.)

D, transverse section passing through the anterior region of a young post-moult scorpion. The rostrum is in the middle of the figure. The accumulation of leucocytes (stained as a dark mass) is seen at its base. The scale represents 270 μ. (Mallory's triple stain.)

E, transverse section passing through the anterior region of the stomodaeal commissure. Note the 'hypocerebral ganglion' attached to the gut, and the ganglionic mass situated above the stomodaeal commissure. The scale represents 75 μ. (Staining as in D.)

F. The same section as in E, enlarged to show the hypocerebral ganglion. The scale represents 31 μ.

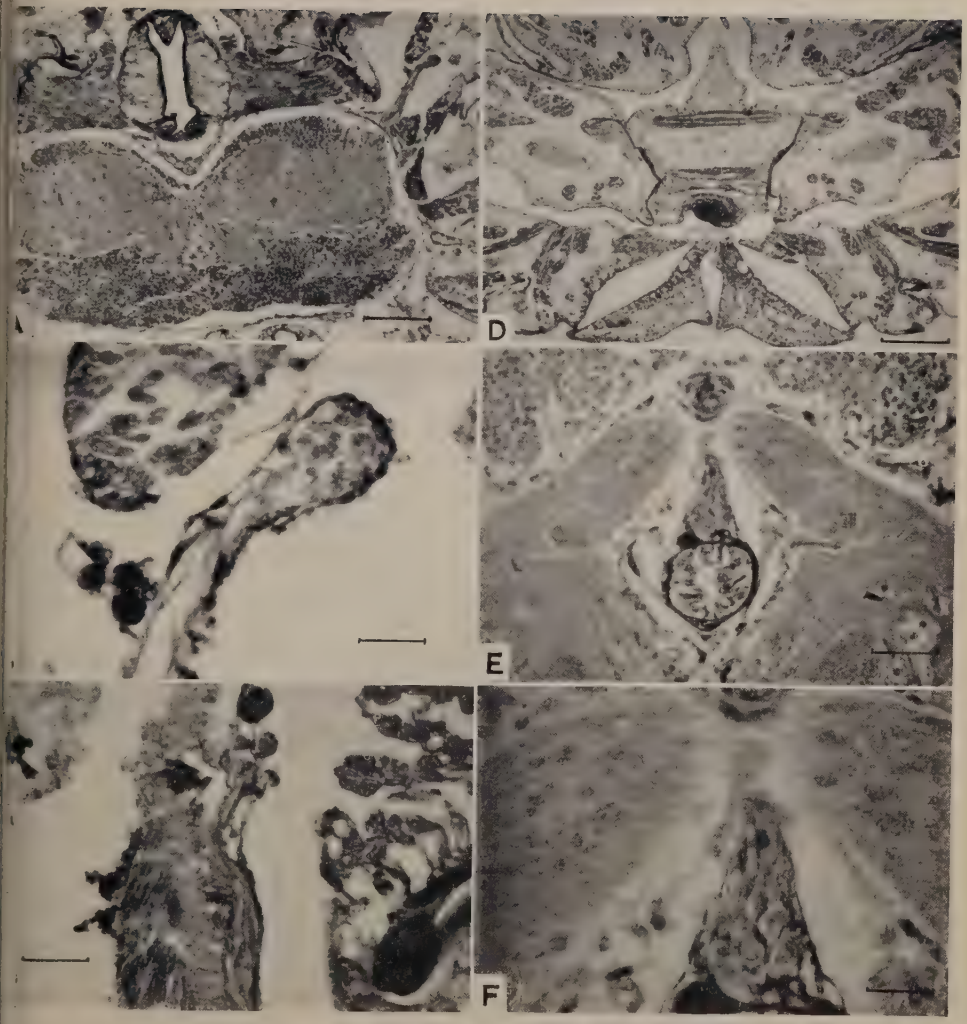

FIG. 2

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(see fig. 3). The origin of the intestinal nerves of Police (Gabe, 1955) from this region also supports this view.

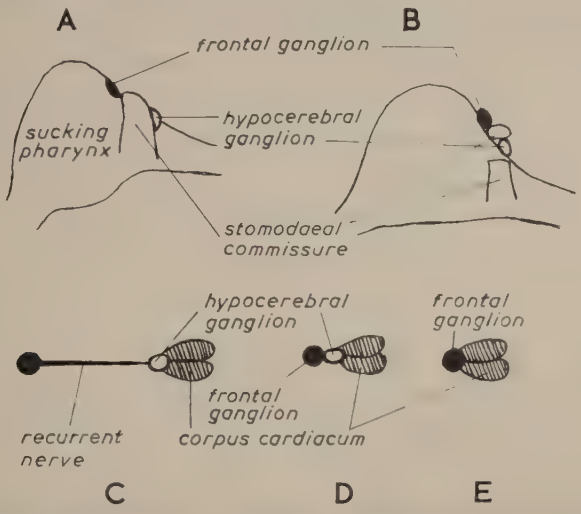

FIG. 3. Diagrammatic representation of supposed homologies between the corpus cardiacum and hypocerebral ganglion of a pterygote insect with the stomatogastric ganglion of the scorpion, *H. swammerdami*. A, a schematic representation of the positions of the ganglionic masses and stomodaeal commissure in the scorpion. B, the same as A, but a part of the stomodaeal commissure is removed to show the positions of the frontal and 'hypocerebral' ganglia. C, D, E, diagrams intended to show how the stomatogastric ganglion of the scorpion can be compared with that of a pterygote insect. C, stomatogastric system of a pterygote insect. D, hypothetical transitory stage. The recurrent nerve is absent. There is fusion of the frontal and hypocerebral ganglia. E, stomatogastric ganglion of an adult scorpion. The recurrent nerve is absent. The hypocerebral ganglion either disappears or fuses beyond recognition, and the corpus cardiacum merges with the frontal ganglion, to produce a composite structure.

I am very grateful to Dr. Kandula Pampapathi Rao, Professor and Head of the Zoology Department at Sri Venkateswara University, for suggesting the problem and for his constant help and guidance throughout the course of this investigation.

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On the Identity of the 'Neurofibrils', 'Nissl complex', 'Golgi Apparatus', and 'Trophospongium' in the Neurones of Vertebrates

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With six plates (figs. 2 to 7)

Summary

1. Classical histological methods demonstrate 4 cytoplasmic networks in fixed vertebrate neurones: 'neurofibrils', 'Nissl complex', 'Golgi apparatus', and 'trophospongium'. The work described in this paper was undertaken to find out whether the 4 networks of classical histology correspond to 4 structures recognizable as such in the living neurone, or to only one structure, which may be coloured in characteristic ways by the classical methods.

2. A single continuous network, comprising features traditionally associated with the four classical networks of the fixed cell, can be isolated by micro-dissection and detected by interference microscopy in living vertebrate neurones.

3. When living neurones are centrifuged at a moderate angular velocity, a single continuous network remains visible under the interference microscope. There does not appear to be enough clear space left for 3 other voluminous structures.

4. When living neurones are centrifuged at a high angular velocity, a single continuous network is pushed to the centrifugal pole of the cell. The remainder of the cell then contains only small separate objects.

5. A single continuous network can be detected by interference microscopy in the cytoplasm of fixed, unstained neurones. When the same cells are dyed by a Nissl method, the cytoplasmic network seen in the unstained cells becomes deeply coloured. When the same cells are bleached and then silvered by a Golgi method, the objects that before had bound the dye now are blackened by the reduced silver. The same effect is obtained when the Nissl method is used after silvering.

6. It is inferred from the results of these experiments that there is only one cytoplasmic network in living normal adult vertebrate neurones. This network is demonstrated in fixed neurones of the same type with varying degrees of faithfulness by the classical methods. It is suggested that the terms 'neurofibrils', 'Nissl complex', 'Golgi apparatus', and 'trophospongium' be abandoned.

Contents

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INTRODUCTION

SCHULTZE (1870), Nissl (1888, 1894), Golgi (1898 *a*, *b*), and Holmgren (1899, 1901) described networks in the cytoplasm of fixed vertebrate neurones; these networks are generally known as 'neurofibrils', 'Nissl complex' (or 'bodies', 'network', 'substance', &c.), 'Golgi apparatus', and 'trophospongium'. We shall use these terms in the present paper to denote, in fixed vertebrate neurones, objects that are essentially similar in structure to those described and illustrated by the authors quoted above. The terms 'classical networks' and 'classical methods' will be used to refer to these 4 objects collectively, and to describe the techniques traditionally used for their demonstration in fixed cells.

There is not general agreement about the identity of the classical networks. They cannot be seen clearly in living vertebrate neurones by phase-contrast microscopy, or after vital dyeing (Covell and Scott, 1928; Parat, 1928; Baker, 1944, 1949, 1957 *a*, *b*, 1959; Casselman and Baker, 1955; Thomas, 1948; Malhotra, 1958, 1959; David and Brown, 1961*a*; and others). But Malhotra (1959, 1960*a*) has recently demonstrated a cytoplasmic network in certain vertebrate neurones, examined in the living state under the interference microscope. He concluded from further work that this single network represented both the Golgi apparatus and the Nissl complex of the fixed cell. We have confirmed Malhotra's demonstration of a cytoplasmic network by interference microscopy in different types of living vertebrate neurones (David and Brown, 1961 *a*, *b*), and have developed methods of dyeing this network in living neurones (David and Brown, 1961*c*). In living neurones of the dorsal root ganglia and ventral horns of the spinal cord of young cats and rabbits, the cytoplasmic network that can be seen by interference microscopy is a complex structure, composed of 'bodies', strands, and spaces; it has many of the features that are traditionally associated with the classical networks (David and Brown, 1961 *a*, *b*, *c*). Now that suitable methods for the microscopical identification of a network in living neurones have been found, it seemed worthwhile to return once again to the problem of the identity of the classical networks. The problem is essentially this: either the classical methods demonstrate 4 structures—recognizable as such in the living neurone—or only one structure, which may be altered in characteristic ways by the different classical methods. It is not necessary in this paper to consider at length the many variations in the appearance of the classical networks demonstrable in the cells chosen for this work, nor is it desirable at this stage to review the immense literature that has been accumulating steadily for more than 90

years, or to take sides in the innumerable controversies that have sprung from attempts to study these contentious objects. Yet it is important to state clearly what are the essential morphological characteristics by which we traditionally identify the classical networks.

The evidence presented in this paper about the identity of the classical network is based on two types of experiments: experiments in which we tried to separate the cytoplasmic network of living neurones into parts or constituents, by micro-dissection and by centrifugation of whole neurones at various speeds; and experiments in which individual fixed neurones were successively treated by as many of the classical methods as possible, in an attempt to find out whether fundamentally the same structures were demonstrated by the various methods.

The Four Networks of Classical Histology

Neurofibrils

These form a continuous system of smooth strands, generally less than 1 μ in diameter; in a given neurone, separate fibrils sometimes can be traced from the dendrites, through the perikaryon, to the extremities of the axon. Fibrils often cross one another, or anastomose into dense networks, especially within the perikaryon (Ramón y Cajál, 1909).

Though Schultze (1870) first detected neurofibrils in unstained neurones that had been teased after maceration in blood-serum 'containing a little iodine' (fig. 1), they are most easily seen in neurones that are silvered in special ways, frequently after treatment in strong bases (e.g. Ramón y Cajál, 1903; del Rio Hortega, 1921; Ramón y Cajál and Castro, 1933; Gibson, 1950; Greenfield and Daniel, 1950; Herrera, 1954).

Nissl complex

This is a complicated structure, composed of at least two types of objects. Some are irregular bodies with indistinct margins, often round, triangular, or fusiform; sometimes the bodies are quite small, but massive objects, more than 5 μ long and almost as broad and thick, are often seen. The second type of object consists of extremely thin strands, about 0·5 μ in diameter, generally peppered with granules that are too small to be measured under the light microscope. The strands and bodies are so oriented that the bodies frequently give the impression of being swellings at the nodal points of a complex three-dimensional reticulum. The bodies and granular strands are seen only in the perikaryon and the proximal parts of the larger dendrites; the axon, even in its cone of origin, does not contain these objects (though strands that are not dyed by Nissl's method can be seen there). Clear platanomorph spaces (see p. 486) are sometimes seen associated with the strands.

Nissl dyed these objects in methylene blue, used in a special way (detailed instructions are given in his paper of 1894), but any basic dye can be used if certain precautions are observed. The Nissl complex is basiphil solely because it contains a high concentration of ribonucleic acid (RNA); therefore, any

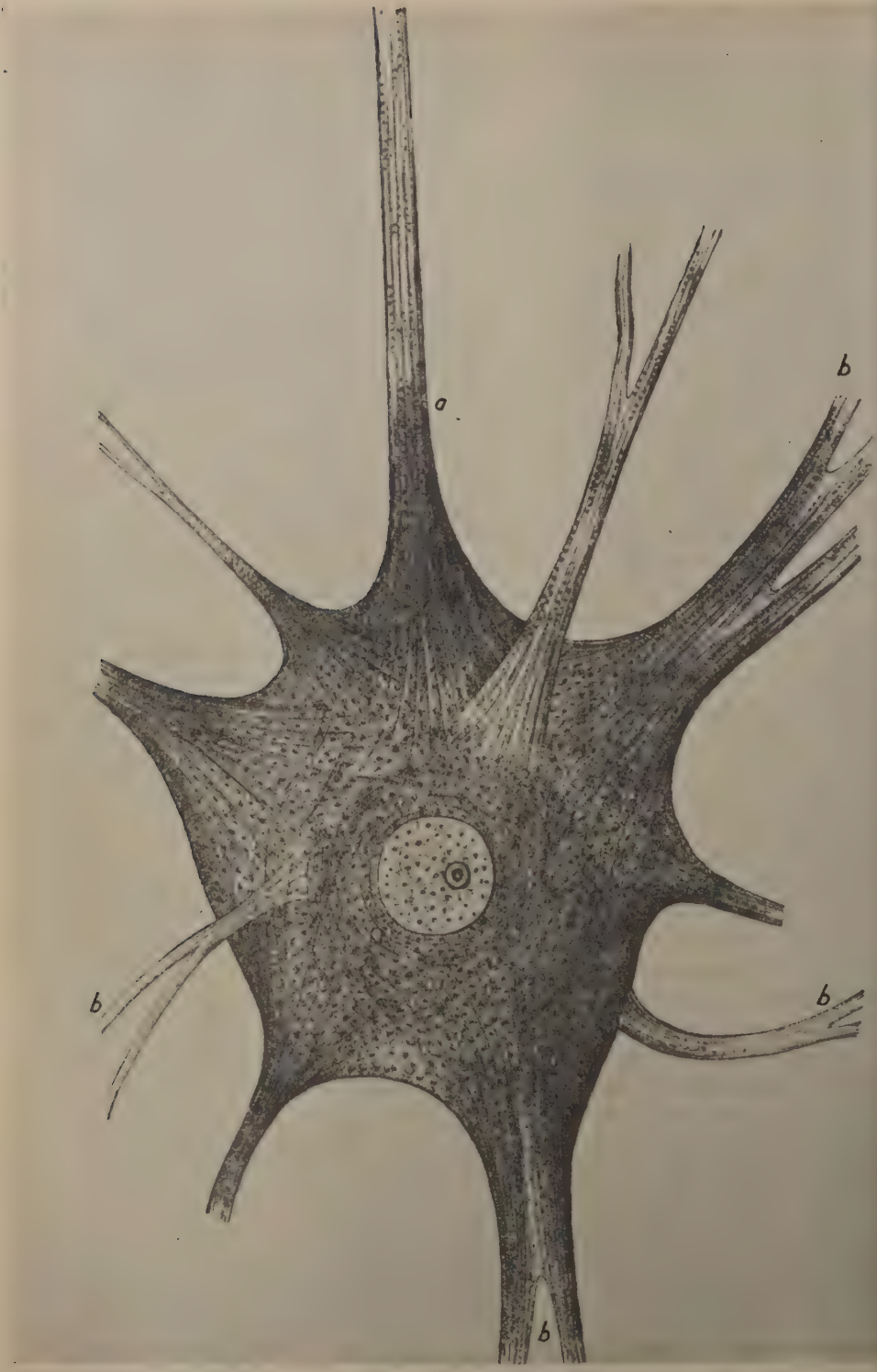

FIG. 1. Neurofibrils in a neurone of the electric lobe of *Torpedo*, isolated after maceration in iodized serum. Reproduced from Schultze (1870).

method that produces a stable coloured compound with RNA will do, and any method that fails to preserve RNA will prevent the subsequent demonstration of the complex (David and Brown, 1961 *a*, *c*). Unfortunately, RNA is dissolved by the pre-treatments used in the methods to silver neurofibrils.

Nissl (1894, 1895) devised a system of classifying neurones on the basis of the intensity of dyeing and shape of the complex. It is not proposed to discuss this system at length (see Einarson, 1933; Vraa-Jensen, 1956), but it is necessary to mention a few points. Nissl recognized three basic shapes of the network or complex: (i) *stichochrome*, characterized by massive bodies that fill the cytoplasm almost entirely, as in the 'motor' neurones of the ventral horns of the spinal cord; (ii) *arkyochrome*, in which interlacing strands support smaller bodies at their nodal points, as in some neurones of the dorsal root ganglia, but most pronounced in the Purkinje neurones of the cerebellum; and (iii) *gryochrome*, characterized by innumerable small globular bodies and extremely thin strands, as in most neurones of the dorsal root and sympathetic ganglia. The Nissl complex of most neurones has a form somewhat intermediate between the fundamental types described by Nissl (Nissl, 1894, 1895; Ramón y Cajál, 1909; Einarson, 1933; Vraa-Jensen, 1956). A neurone of the ventral horns of the spinal cord of the cat is illustrated in fig. 2. This is a series of optical sections through a stichochrome/arkyochrome neurone, fixed in FAM (David, 1955), double-embedded in collodion and paraffin, cut at 7 μ, and dyed in cresyl violet.

Golgi apparatus

In its classical site, the Purkinje neurones of the owl, this structure resembles very closely the Nissl complex described above, with the sole difference that the strands of the Golgi apparatus extend into the axon (see Golgi, 1898*a*, especially his fig. 2). In a later paper, Golgi (1898*b*) illustrated the 'internal reticular apparatus' of neurones of the dorsal root ganglia of the cat; in these he apparently saw only anastomosing strands.

Golgi's original technique was based on his earlier method of silvering whole neurones (Golgi, 1886). This is an awkward method, and it has been superseded by silvering after fixation in formaldehyde containing 'adjuvants' (Golgi, 1908; Ramón y Cajál, 1914; Da Fano, 1920; Aoyama, 1929), and by methods of osmication or post-osmication (Kopsch, 1902; Weigl, 1910; Baker, 1944). Networks resembling Golgi's original drawing (Golgi, 1898*a*, fig. 2) are more readily obtained by our method of silvering paraffin sections (David, Mallion, and Brown, 1960). An example is given in fig. 3. It should be noted that the Golgi apparatus cannot be demonstrated in tissues that have been treated in strong bases, as is done in most methods for the demonstration of neurofibrils (Golgi, 1908; Legendre, 1910).

Trophospongium

This consists of separate spaces, generally in the shape of a banana or a sausage, between 0·2 and 2·0 μ in cross-section and up to about 5 μ long.

Sometimes three or more spaces are fused, giving the appearance, in section, of a maple-leaf. However, all the spaces within the cytoplasm of a mammalian neurone do not anastomose to form a canalicular system. The terminology of these objects is confusing. Holmgren gave the name *trophospongium* to spaces detected in neurones of the dorsal root ganglia of the teleost *Lophius piscatorius* (Holmgren, 1899); later, he used the same name to denote the spaces seen in the cytoplasm of mammalian neurones (Holmgren, 1901). This was unfortunate, since the objects he saw in the neurones of *Lophius* were invaginations of the cell membrane (David, unpublished observation), similar to those seen in neurones of some invertebrates (Wigglesworth, 1959; Malhotra, 1960*b*). For this reason, we have suggested a purely morphological term, platanomorph (= banana-shaped) spaces (David and Brown, 1961*a*). Holmgren homologized the trophospongium with the Golgi apparatus, and developed an extremely complicated theory to explain its function. The term trophospongium will be used in this paper to denote objects similar to those seen by Holmgren in mammalian neurones, that is to say platanomorph spaces; the meaning 'invaginations of the cell-membrane' will not be used.

It is not necessary to use special methods of fixation or colouring in order to demonstrate the trophospongium; it is identified because it remains a set of colourless transparent objects when the rest of the cytoplasm is coloured. There is no known way of colouring these objects positively, though some methods of silvering occasionally blacken the *surface* of the spaces (see Malhotra, 1959). The trophospongium is clearly visible in the photomicrographs of neurones silvered by a Golgi method illustrated here (fig. 3).

Micro-dissection of Living Neurones

In preliminary experiments (David and Brown, 1961 *a*, *b*, *c*) we found that the cytoplasm of living vertebrate neurones was so crowded with all sorts of inclusions that a comparatively unobstructed image of the network could not be obtained in whole neurones, even when they were flattened in a compressorium. We therefore attempted to remove the obstructions by mechanical means in order to obtain the clearest possible view of the network under the interference microscope.

The most successful results were obtained with dendrites of the large stichochrome neurones of the spinal cord of the cat. The technique is very simple. Neurones are isolated free-hand and cleaned in a drop of saline, by the method described in previous papers (David and Brown, 1961 *a*, *b*). They are then examined uncovered at a magnification of about 200 diameters, under a dissecting microscope; a suitable dendrite is gently manipulated to the centre of the field. The cell membrane surrounding the dendrite is carefully slit with the aid of a triangular knife, made by bending and trimming with iridectomy

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Fig. 2 (plate). Optical sections through a neurone of the ventral horn of the spinal cord of the cat, illustrating the Nissl complex. This cell was fixed in FAM, dehydrated in methanol, double-embedded in collodion and paraffin, sectioned at 7 μ, dyed in cresyl violet, and photographed in monochromatic light of 546·1 mμ (David, 1955).

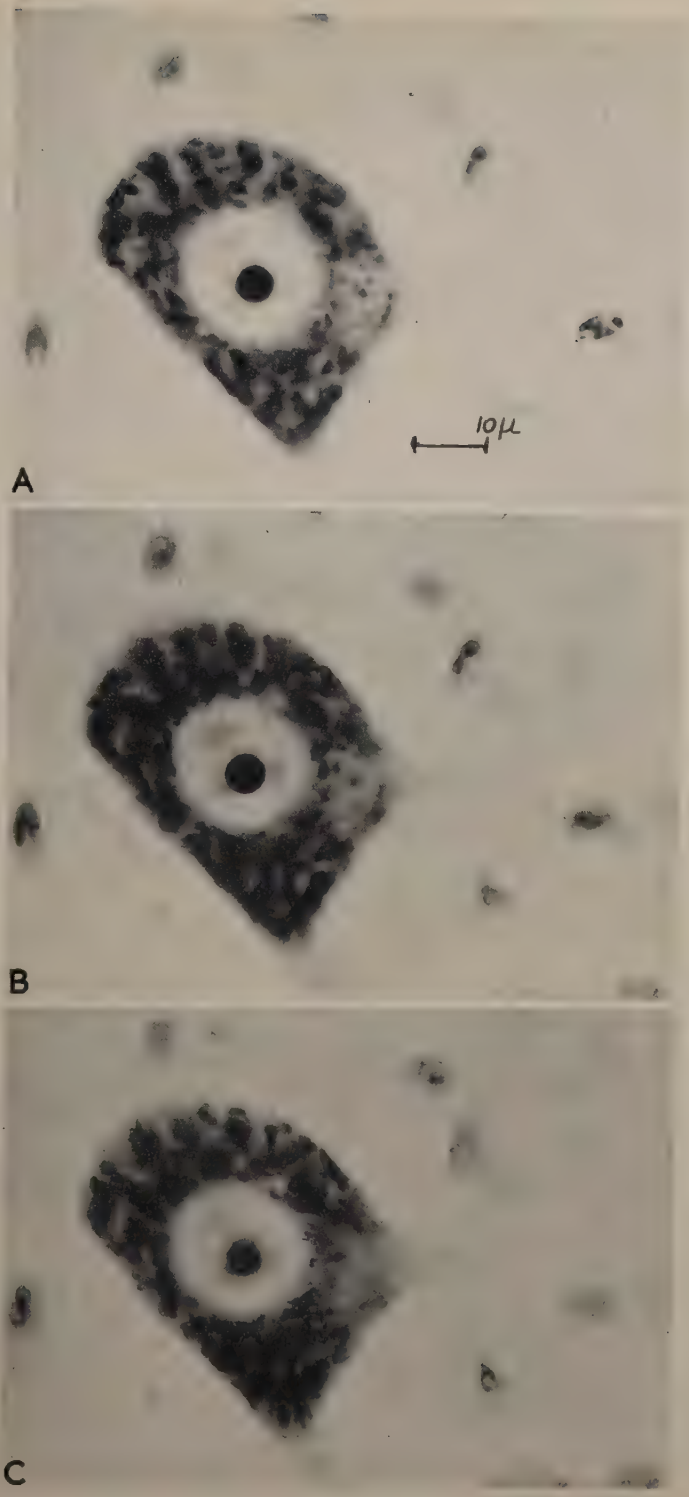

FIG. 2

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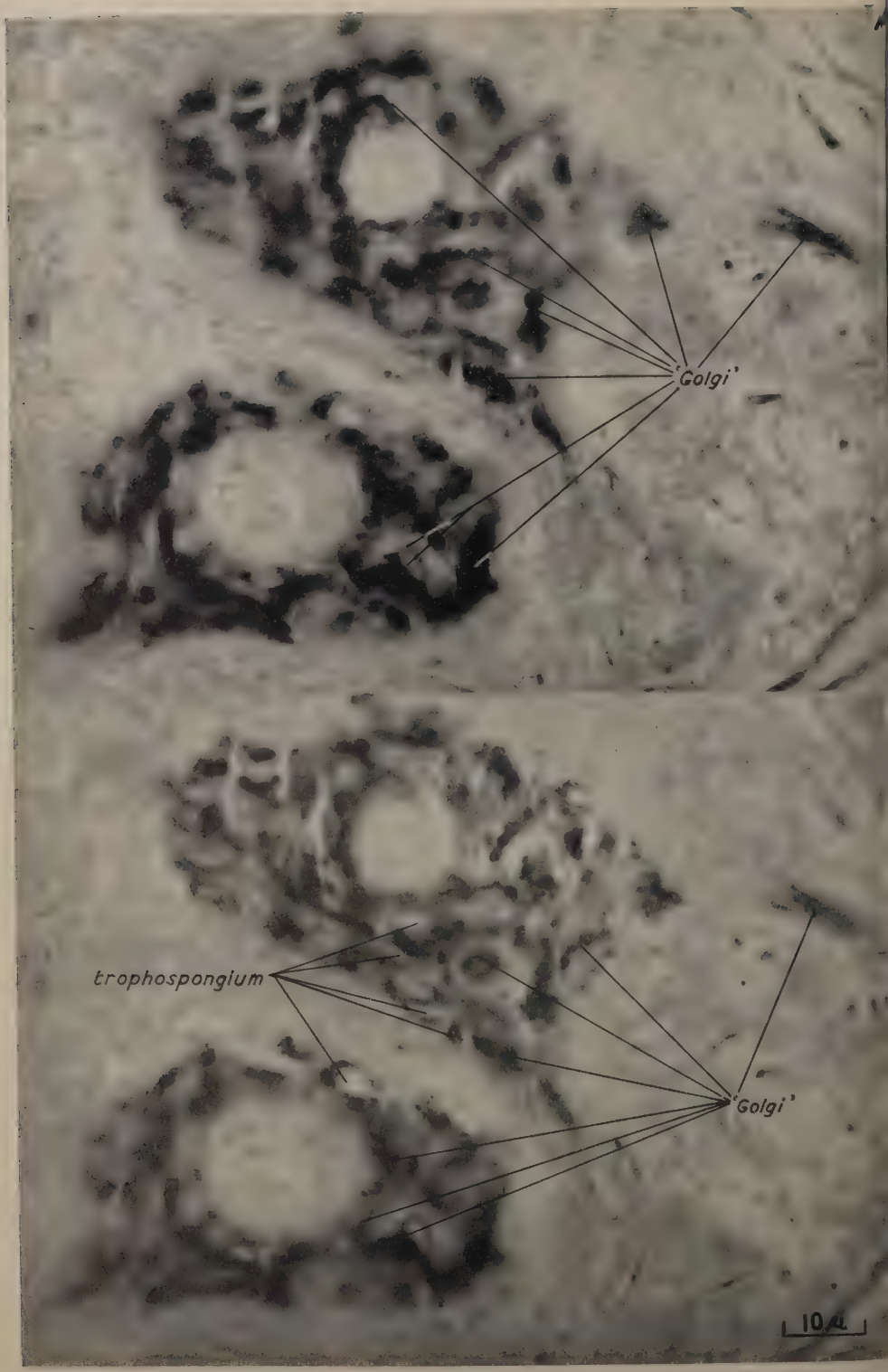

FIG. 3

G. B. DAVID, A. W. BROWN, *and* K. B. MALLION

scissors a length of stainless steel wire, 18 μ in diameter. The cell then can be examined immediately, or further teasing can be attempted with a split hair or a thin needle. For most purposes it is best to cover the cell and examine it at once under the 2-mm shearing objective of the Smith interference microscope.

When the cell membrane is slit, any separate objects within the dendrite escape into the surrounding fluid. The cytoplasmic network then stands out sharply against a clear background (fig. 5, A, facing p. 489). This isolated network appears to be composed of two objects: dense, rather granular strands, about 0·5 μ in diameter, crossing one another and eventually anastomosing, though frequently stacked in parallel bundles for long distances; and clear platanomorph spaces, about 1 to 2 μ in cross-section and 3 to 10 μ long, occasionally anastomosing in twos and threes, but not in communication with the exterior of the cell. The nodal points of crossing and anastomosing strands frequently give the impression of being irregular bodies with indistinct margins.

It was not found possible to subdivide the network into separate components by any amount of teasing. At best, some of the marginal strands could be made to snap, and then spring up into the surrounding fluid (fig. 5, A). No amount of prodding could dislodge the platanomorph spaces, or unravel crossed strands. The strands of the network illustrated in fig. 5, A bear a surprisingly close resemblance to the neurofibrils of Schultze's (1870) original drawing (fig. 1, p. 484).

Dendrites prepared as described above deteriorate rapidly. Within 20 min, artificial vacuoles appear between some of the strands. The vacuoles then swell, forcing the strands to coalesce into 3 or 4 massive ribbons. At the same time, the outer strands undergo a curious process of clasmatosis, almost as if the granules on their surface had suddenly swollen into large pear-shaped hollow structures, surrounded by layers of desquamating 'membranes'. Once one has seen a deteriorating preparation, it is not easy to mistake it for the structure of a dendrite in good condition.

The Effects of Centrifuging Living Neurones

General remarks

It was thought that new facts about the cytoplasmic network might be revealed if neurones were examined in the living state after centrifugation. The contents of intact vertebrate neurones only stratify when centrifuged at much higher angular velocities than are required for other types of cells (Ingvar, 1923; Beams and King, 1935; Brown, 1936; Beams and Kirshenblit, 1940). There is not general agreement about the causes of this (see Ingvar, 1923; De Rènyi, 1932). We have carried out two types of experiments. In the first of these, neurones were centrifuged at angular velocities that are high enough to

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Fig. 3 (plate). Optical sections through a pair of neurones of the ventral horn of the spinal cord of the cat, illustrating the Golgi apparatus and the trophospongium. These cells were fixed in FAM, dehydrated in methanol, double-embedded in collodion and paraffin, sectioned at 7 μ, and silvered by a Golgi method (David, Mallion, and Brown, 1960).

stratify the separate cytoplasmic inclusions of other types of cells; it was hoped either to shift the network to one pole of the neurone, leaving the separate inclusions distributed as before, or to shift these while leaving the network intact. In the second, neurones were subjected to prolonged centrifugation at the highest angular velocities obtainable with the equipment at our disposal. It was hoped that the neuronal constituents would become fully stratified, and that any heterogeneity between parts of the network would become apparent.

Both types of experiment were carried out with neurones of the dorsal root ganglia of the cat and rabbit. The method of centrifuging has been described in another paper (David and Brown, 1961 *d*). After centrifuging, the neurones were teased in a drop of a saline solution (Baker, 1944) containing 20% w/v crystallized bovine plasma albumin, mounted, and examined at once under the 2-mm shearing objective of the Smith interference microscope. Other neurones were fixed for further work.

It was found that centrifugation caused very little damage to the neurones. The stratification of the cellular contents is reversible, and even fully stratified cells were found to become indistinguishable from untreated ones within 15 min. For this reason it is important to work very quickly.

Experiments at moderate angular velocities

It was found that centrifugal forces of less than 45,000 g had no effect on the neurones that could be detected by any method of subsequent vital examination. A curious effect could be seen in the neurones that were centrifuged at 45,000 g for 30 min, and then examined under the interference microscope (see fig. 4).

The neurones now become somewhat elongated. The nucleus (not visible in fig. 4) remains central, or nearly central. It is extremely difficult to determine the direction of the centrifugal force by examining the teased neurones. The cytoplasmic network appears with surprising clarity, yet the separate inclusions appear not to have shifted much from their original sites. The network is largely made up of anastomosing granular strands, less than 0·5 μ in diameter. The strands seem to be arranged concentrically within the cytoplasm; bundles of strands run a parallel course, converging and anastomosing here to form a spidery body, diverging there to leave a clear platanomorph space. Where the strands run a parallel course, the distance between them varies between about 0·2 and 0·8 μ. The cytoplasmic network does not absorb light of the wavelength used with the interference microscope (the mercury green line of 546·1 mμ). It could be considered to be a pure phase-grating, with retarding and non-retarding segments of approximately the same width (between $\frac{1}{2}$ and $1\frac{1}{2}$ wavelength). This type of object is of great theoretical interest, and it adds special problems to the interpretation of the images obtained under the interference microscope. (This will be discussed in a later paper.)

FIG. 4 (plate). Living neurone of a spinal ganglion of the cat, photographed at 546·1 mμ under the 2-mm shearing objective of the interference microscope after centrifugation at 45,000 g for 30 min at 2° C.

FIG. 4

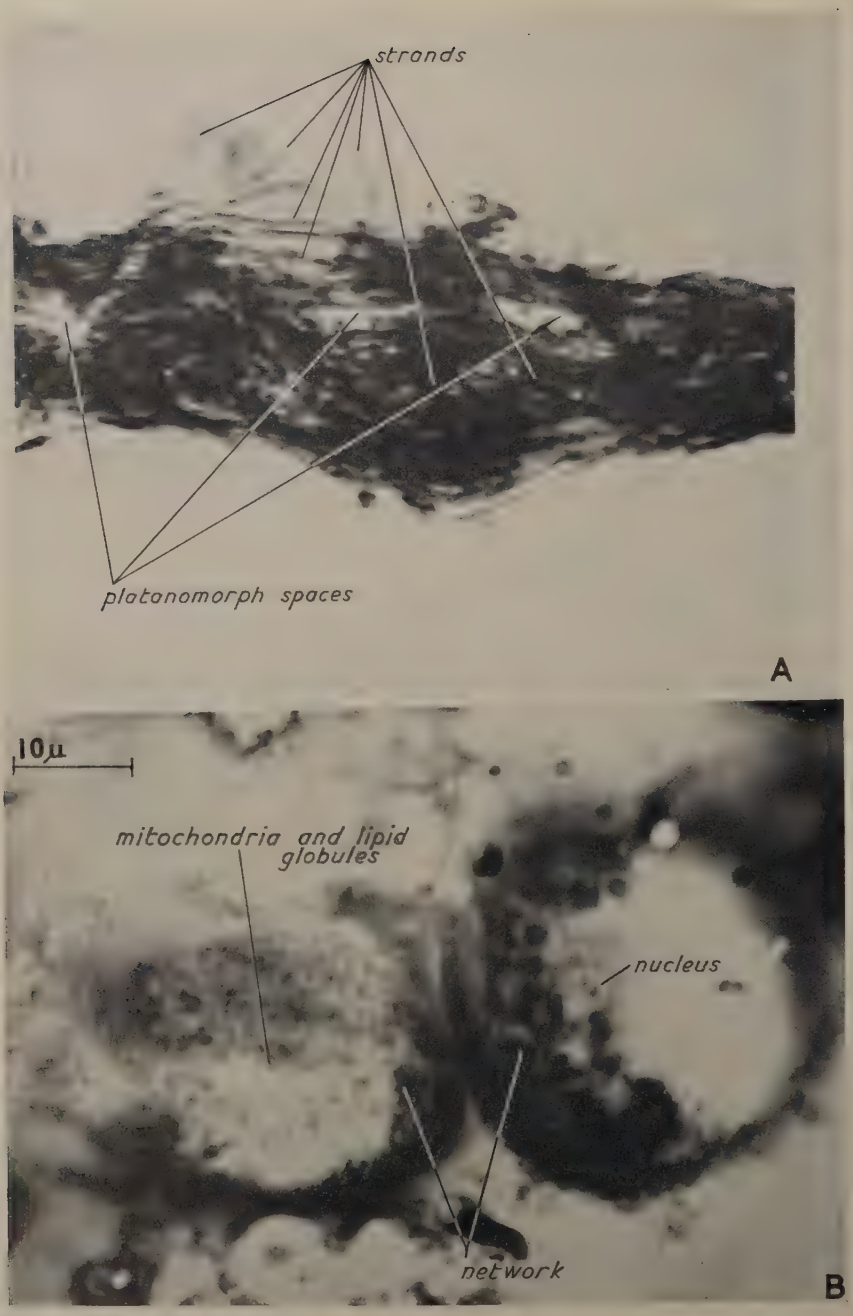

FIG. 5

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Experiments at high angular velocities

We found empirically that the contents of living vertebrate neurones would stratify after centrifugation for 3 h at 95,000 g (fig. 5, B). The cytoplasmic network moves centrifugally, as do the nucleus and the plasmosome within the nucleus. Mitochondria appear not to move. Lipid globules move centripetally.

The stratified cytoplasmic network is seen under the interference microscope even more clearly than before. Now, the bodies at the nodal points of the strands are more sharply outlined: they are seen to be spidery centres from which strands radiate in all directions; the bodies are much more numerous than before. The strands give the impression of being shorter and thicker. These changes are probably due to the compression of the network into a much smaller volume than that occupied before centrifugation. The platanomorph spaces move with the rest of the network.

The centripetal part of the cell contains only small separate objects, that is to say mitochondria and lipid globules. These are seen rather more clearly than in untreated neurones. We know of no method that allows a clearer view of the filamentous mitochondria of vertebrate neurones. The whole of the cytoplasmic network is contained within the centrifugal part of the cell. This was confirmed by examining fixed, unstained sections of centrifuged ganglia under the 2-mm double-focus objective of the Smith interference microscope (fig. 6). Only three types of objects could be seen in the centripetal part of the cell: filamentous mitochondria, about 0·2 μ in diameter and 1 to 4 μ long; homogeneous refractile globules, spherical or nearly spherical, and about 0·5 μ in diameter; and compound globules, about 0·8 to 1·2 μ in diameter, made up of a refractile externum and an internum of comparatively low optical density.

Successive use of Several Classical Methods on the Same Set of Vertebrate Neurones

The basic method of investigation was developed by Malhotra (1959, 1960*a*). Neurones are coloured by a classical method, photographed, bleached, coloured by another classical method, and photographed again. We chose to work with the large stichochrome neurones of the spinal cord of the cat. These cells were chosen in preference to arkyochrome neurones because it was thought that it would be easier to recognize the few massive constituents of their cytoplasmic network after different methods had been applied, than the smaller, more complicated structures of arkyochrome neurones. Since techniques of silvering neurofibrils require special fixation, and are incompatible with the subsequent application of Golgi or Nissl methods, only the identity

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Fig. 5 (plate). A, dendrite of a living neurone of the ventral horn of the spinal cord of the cat, photographed at 546·1 mμ under the 2-mm shearing objective of the interference microscope after the cell membrane had been stripped off by micro-dissection.

B, pair of living neurones of a dorsal root ganglion of the cat, photographed at 546·1 mμ under the 2-mm shearing objective of the interference microscope, after centrifugation at 95,000 g for 3 h at 2° C.

of the Nissl complex, Golgi apparatus, and trophospongium was considered in the experiment described below.

Pieces of cat spinal cord were fixed in FAM (David, 1955), dehydrated in methanol, double-embedded in collodion and paraffin, and carefully sectioned at 7 μ (David and Steel, 1959). The Golgi apparatus was silvered by a method applicable to paraffin sections (David, Mallion, and Brown, 1960), and bleached in Farmer's ferricyanide/thiosulphate reagent (Farmer, 1883, 1884). The Nissl complex was dyed in monomeric cresyl violet (David, 1955), and bleached in Gothard's reagent (Gothard, 1898).

The untreated sections were mounted in anhydrous glycerol and examined with the 2-mm double-focus objective of the Smith interference microscope. Suitable neurones were photographed. The sections then were taken down to water, divided into groups, and processed as follows. Some were first silvered, then bleached, then dyed; others were first dyed, then bleached, and then silvered. The neurones that were first photographed unstained with the interference microscope, were photographed again (by direct microscopy) after silvering and dyeing. They were also examined after each bleaching procedure, to make sure that all the dye or silver had been removed.

A cytoplasmic network, fundamentally similar in structure to that seen in living neurones, can be detected by interference microscopy in the fixed unstained neurone (fig. 7, A). When the same neurone is silvered by a Golgi method, the entire cytoplasmic network blackens (fig. 7, B). Therefore, the network seen in fig. 7, A can legitimately be called a Golgi apparatus. When the same neurone is bleached and then stained by a Nissl method, the whole of the network that had been blackened by the reduced silver before now binds the basic dye (fig. 7, C). Therefore, the network seen in fig. 7, A can also be called a Nissl complex and, inescapably, Golgi apparatus and Nissl complex are, in this instance, only two names for a single structure. When this experiment is performed in the inverse sense, that is to say when the Nissl method is used before the Golgi, the results are precisely the same: the basiphil network is also blackened by silver. The trophospongium is not affected by Nissl or Golgi methods: in both cases it appears as platanomorph spaces invariably associated with strands or bodies of the network.

Discussion

The results of the experiment on micro-dissection (pp. 486–7) indicate that the cytoplasmic network detected in living vertebrate neurones by interference microscopy is a single, continuous structure. This structure seems to have great mechanical strength, since it could not be broken up by available

Fig. 6 (plate). Neurones of the dorsal root ganglia of the cat, photographed at 546·1 mμ under the 2-mm double-focus objective of the interference microscope. These neurones were fixed in Lewitsky/calcium (David and Brown, 1961*d*) after centrifugation at 95,000 g for 3 h at 2° C, embedded in gelatine, and cut at 3 μ on the freezing microtome. In A, the analyser was adjusted to secure minimum illumination of the network; in B, minimum illumination of the background.

FIG. 6

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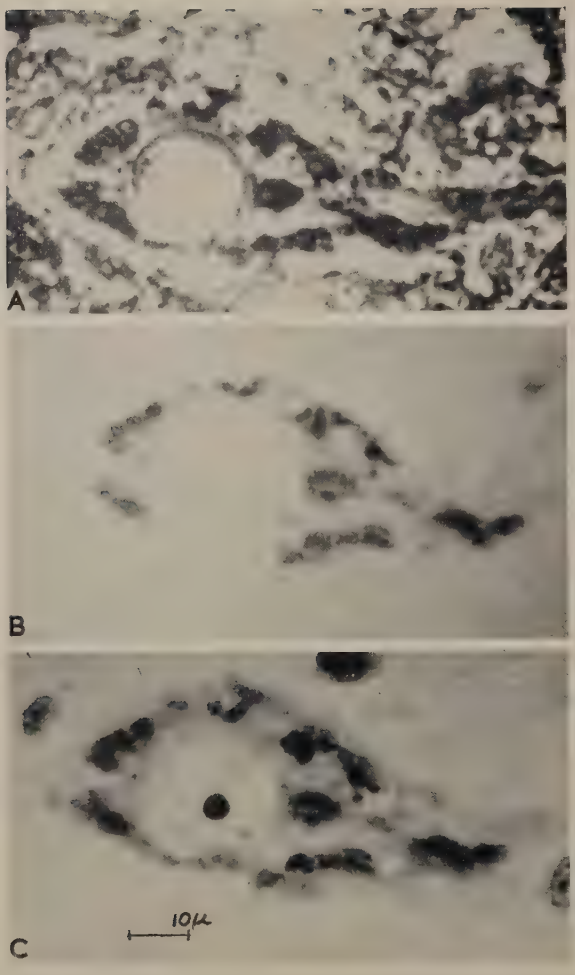

FIG. 7

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methods of micro-dissection. It is made up of interlacing strands, with associated granules and platanomorph spaces. The results of the experiment in which neurones were lightly centrifuged (pp. 487–8) support these conclusions. At no place in the centrifuged neurones can we see a discontinuity large enough to suggest that there might be more than one network. Further, the continuous network that we see is so massive, and leaves so few gaps in the cell, that we are forced to conclude that there simply is no room left for any other voluminous structures. Centrifugation at high angular velocities (p. 489) pushes a single continuous network to the centrifugal end of living vertebrate neurones. The remainder of the cell then contains only small separate objects. These findings can leave little doubt that the cytoplasmic network seen in living neurones is a single structure.

The experiments in which neurones were centrifuged generally confirm earlier work in which centrifuged neurones were only examined after treatment by classical methods (e.g. Beams and King, 1935; Beams and Kirshenblit, 1940). The interpretations of Brown (Brown, 1936), however, are not compatible with our findings. Brown suggested that the Golgi apparatus moved centripetally, the Nissl complex centrifugally, and the trophospongium did not shift. It is probable that what Brown termed 'Golgi apparatus' was an artifact, caused by unspecific deposits of osmium and silver on and between the separate lipid globules at the centripetal pole of the cell. These artificial networks have been shown to form in neurones of the same type (Baker, 1944; Thomas, 1948). Brown's inability to shift the trophospongium by centrifugation (Brown, 1936) cannot be explained.

The single continuous network seen in the living vertebrate neurone is equally prominent under the interference microscope in fixed, unstained neurones (see fig. 7, A). We suggest that the 4 classical networks represent this single structure, or part of it. Taking the simplest possible case, it is clear that the trophospongium of most vertebrate neurones is nothing other than the platanomorph spaces that are seen between some of the strands of the network. The results of the experiments in which individual neurones were treated by several classical methods in succession (pp. 489–90 and fig. 7) fully confirm Malhotra's finding that the Golgi apparatus and the Nissl complex were only one structure (Malhotra, 1959, 1960*a*). They also suggest that Nissl methods colour the granules associated with the strands, while Golgi methods blacken both granules and strands; presumably this is the reason why the strands of the axon are demonstrated by Golgi and not by Nissl methods. The neurofibrils of classical histology are very similar to the strands that are seen by interference microscopy in dendrites of living neurones subjected to micro-dissection (see figs. 1, p. 484, and 5, A, facing p. 489).

FIG. 7 (plate). Three photomicrographs of the same neurone of the ventral horns of the spinal cord of the cat, fixed in FAM, dehydrated in methanol, double-embedded in collodion and paraffin, and sectioned at 7 μ. In A the neurone was photographed unstained with the interference microscope, immersed in glycerol; in B after silvering by a Golgi method; in C after bleaching the silver and dyeing by a Nissl method.

If, as suggested by Malhotra (1959), the cytoplasmic network of living neurones can be homologized with the endoplasmic reticulum seen in fixed cells of the same type by electron-microscopy (Palay and Palade, 1955; Palay, 1956; Fernandez-Morán, 1957), it might be reasonable to assume that the neurofibrils are deposits of silver on the distorted remnants of the 'membranes' or 'tubules' of the reticulum. The Palade granules of the reticulum (Palade, 1955) contain much RNA, and they are undoubtedly capable of binding basic dyes to produce the Nissl complex; but they could hardly be preserved by the pre-treatments of the methods used to demonstrate neurofibrils. However, platanormorph spaces are not visible in electron micrographs of the endoplasmic reticulum (Malhotra and Meek, 1960); certain objects seen in electron micrographs, and usually termed 'non-granular reticulum' (Palay and Palade, 1955), do not appear to correspond to any part of the cytoplasmic network seen in living vertebrate neurones.

We should like to end by suggesting that, since the 4 classical networks represent with varying degrees of faithfulness that which is a single structure in living normal adult vertebrate neurones, the terms neurofibrils, Nissl complex, Golgi apparatus, and trophospongium should be abandoned. It is desirable to have an unexceptionable term for this object. The term endoplasmic reticulum seems unsuitable (see Malhotra, 1959, 1960*c*); a simpler and better one may be cytoplasmic network (of vertebrate neurones). The question of homologies must be deferred until it can be put on a firm experimental basis. When it is necessary to refer to the constituents of this network, this can be done unambiguously by using purely descriptive terms such as strands, platanomorph spaces, granules, &c.

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Morphological and Histochemical Studies of the Chromatoid Body and Related Elements in the Spermatogenesis of the Rat

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With three plates (figs. 1 to 3)

Summary

In the spermatogenesis of the rat the chromatoid body is present during the growth of the primary and secondary spermatocytes, disappears at telophase of both the meiotic divisions, and is absent during interkinesis. It is reconstructed during the early stages of spermateleosis but after the elongation and condensation of the nucleus it gradually becomes smaller and disappears. Simultaneously, in the caudal region von Ebner's stainable granules appear and gradually fuse together to form a single voluminous body, Regaud's *sphère chromatophile*, which is discarded with the residual body and is phagocytosed by the Sertoli cell.

The histochemical studies reveal that the chromatoid body, von Ebner's stainable granules, and the *sphère chromatophile* are similar in composition. They consist mainly of RNA and proteins, and this suggests that they may be centres of protein synthesis. The RNA content of von Ebner's stainable granules and the *sphère chromatophile* appears to be higher than that of the chromatoid body. This probably means that there is a progressive decrease in the protein component of the chromatoid material. Also there is a distinct change in the chemical composition of the protein component of the chromatin during the late stages of spermateleosis. It is tentatively suggested that the function of the chromatoid material may be to provide basic proteins for the final maturation of the chromatin of the late spermatid.

It appears that the chromatoid elements originate from the ground cytoplasm and disappear by merging into the latter.

An enigmatic granular satellite has been found associated with the chromatoid body. It differs from the latter in its chemical composition.

Introduction

In my recent papers on the chromatoid body in spermatogenesis I emphasized that this cytoplasmic inclusion needs to be studied by histochemical methods and electron microscopy in order to formulate criteria for its identification and to trace its origin and physiological significance (Sud, 1961 *b*, *c*). In this connexion the histochemistry of the chromatoid body in the grass-snake, *Natrix natrix*, has already been studied and a tentative suggestion has been made about its function (Sud, 1961 *a*, *b*). It was considered desirable to devote the next study to the histochemistry of the chromatoid body in the spermatogenesis of the rat, the species in which it was first discovered and later studied in detail by a large number of prominent workers.

The chromatoid body of the rat was first figured in the young spermatid by von Brunn (1876), and later studied by several authors in fixed cells (Benda, 1891; Moore, 1893, 1894; Niessing, 1897; Lenhossék, 1898; von Ebner,

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1899; Duesberg, 1908, 1909; Regaud, 1910; Allen, 1918; Ludford and Gatenby, 1921; Daoust and Clermont, 1955), in living cells under phase contrast (Austin and Sapsford, 1951), and by electron microscopy (Watson, 1952; Swift, 1956). Except for the remark by Daoust and Clermont (1955) that the chromatoid body in the rat spermatid is deeply stained by pyronin (which suggests that it contains RNA), no attempt has previously been made to study the histochemistry of the chromatoid body in this species.

The present paper includes a report on a detailed histochemical study of the chromatoid body, its granular satellite, the stainable granules (*die tingirbaren Körnchen*) of von Ebner (1888), and Regaud's (1901*b*) *sphère chromatophile*, in the spermatogenesis of the rat. In addition observations have been recorded on the morphology and behaviour of these cytoplasmic inclusions and the results compared with those of previous authors.

Material and Methods

The white rat, *Rattus norvegicus albinus*, was used for the present investigation. Specimens of different ages were tried and it was found most convenient to work with rats weighing about 250 g. In these the testes seem to be most active and show practically all stages of sperm-formation, especially spermateleosis.

The testes were dissected out rapidly, divided into small bits by a sharp razor or a pair of scissors, and the pieces directly transferred to various fixatives. Paraffin wax, collodion, and gelatine were used as embedding media for different histochemical tests as detailed in the appendix (p. 504).

For deamination van Slyke's mixture, introduced into histochemistry by Monné and Slautterback (1950), was used. It consists of 2 volumes of concentrated aqueous sodium nitrite solution, 1 volume of glacial acetic acid, and 5 volumes of distilled water. The preparations were deaminated in the freshly prepared mixture for 12 h at room temperature, washed thoroughly in water, and the deaminated and control preparations subjected to exactly the same treatment.

Fresh material was studied under phase-contrast microscope in 0·9% saline, to every 100 ml of which 0·2 ml of 10% anhydrous calcium chloride had been added (Baker, 1944), or in an isotonic solution of Armour's bovine plasma albumin, fraction V, in 0·75% Baker's saline. Neutral red and Janus green were used supravitally. Both these vital dyes were used by diluting 0·1 ml of a 0·5% aqueous stock solution of the dye with 5 ml of the above-mentioned 0·9% saline. A small piece of the testis tubule was immersed in the diluted solution for 5 to 10 min, teased, and studied by direct microscopy.

Results

Morphological studies

The chromatoid body in the spermatogenesis of the white rat, *R. norvegicus albinus*, appears for the first time during the growth of the primary spermatocyte. It is a homogeneous, spherical body, measuring up to 0·9 μ in diameter

(fig. 1, A; 2, B, C; 3, B). In the large primary spermatocyte there are one to three, generally two, chromatoid bodies, usually lying on opposite sides of the nucleus on or near the long axis of the cell (fig. 2, E). One of these is the main chromatoid body already present in the earlier growth period of the cell, and the remaining one or two are very small additional chromatoid bodies in the form of prominent granules which appear during the later growth of the cell. The chromatoid bodies usually lie at some distance from the nuclear membrane and have no topographic relationship with the idiozome.

During the first meiotic division the chromatoid bodies persist as far as the late anaphase stage, but are absent during interkinesis. During the growth of the secondary spermatocyte the chromatoid body reappears as a single homogeneous sphere measuring up to 1·2 μ in diameter. It often lies close against the nucleus. Once again it is visible up to late anaphase, but later it disappears.

In the spermatid the chromatoid body reappears during the initial stage of acrosome-formation. At the start it lies on one side of the nucleus but later it shifts to its posterior end (fig. 1, B, E.) It may have an irregular outline but ultimately it assumes a spherical form and measures up to 1·8 μ in diameter. Before the centriole spins out the axial filament, a lighter and a darker portion may be distinguished in the chromatoid body in preparations stained with basic dyes, basic dye-lakes, and particularly after the acid haematein test (fig. 1, B), but afterwards it appears homogeneous even in such preparations (fig. 1, E; 2, A). Invariably the chromatoid body in the spermatid is accompanied by a granular satellite which either lies in contact with it (fig. 1, B) or adjacent to it: in the latter case the gap between the two is bridged by faintly stained material (fig. 1, E).

When the cytoplasm shifts behind the nucleus to form the tail-bladder, the chromatoid body is seen adjacent to the nucleus within the *manchette* beside the axial filament. Generally at this stage the granular satellite separates itself from the chromatoid body and lies at a little distance from the latter (fig. 2, A, C). After the elongation and condensation of the spermatid nucleus, while the cytoplasm of the tail-bladder is spreading further back along the axial filament, the chromatoid body undergoes rapid diminution without changing its position (fig. 1, C; 2 E), and ultimately disappears. The satellite apparently becomes confused with the centrioles.

Simultaneously with the disappearance of the chromatoid body, the stainable granules of von Ebner (1888) appear in the form of numerous granules filling the caudal region of the spermatid. These granules flow together into a single large spherical body called by Regaud (1901*b*) the *sphère chromatophile* (fig. 1, D; 2, F). The latter gradually shifts to the front end of the head of the maturing spermatozoon and becomes frothy and voluminous, assuming an irregular contour (fig. 1, E; 2, G; 3, F). The cytoplasm containing this body is detached from the spermatozoon to form the residual body. The residual bodies containing the *sphère chromatophile* form a zone between the spermatozoa and the spermatogenetic cells, and they remain in this position for some time after the spermatozoa are shed. The residual bodies remain connected

with the Sertoli cells, and the *sphère chromatophile* enclosed in each of them appears to pass to the base of the Sertoli cell (fig. 1, E). Some of the residual bodies, however, are not phagocytosed but are left behind in the lumen, where they degenerate.

The chromatoid body in the young spermatid, when studied in living material under the phase-contrast microscope, gives a high phase-change and appears slightly darker than the idiozome (fig. 3, G). Neutral red and Janus green, used supravitally, leave it untouched.

Histochemical Studies

In the primary spermatocyte even the larger chromatoid body is small in comparison with the size of the cell and therefore it is not invariably seen in sections. In the secondary spermatocyte it is slightly bigger than that of the primary spermatocyte but the cell is not of common occurrence since the life of the secondary spermatocyte only amounts to about 2% of the spermatogenetic wave (Roosen-Runge and Giesel, 1950). In the young spermatid, however, the chromatoid body assumes the largest size; in addition the cell is small, and the body therefore generally appears in sections. For this reason the chromatoid body at the young spermatid stage of the rat lends itself best to histochemical study. At this stage it is possible to find it even in preparations showing negative results, while in the spermatocytes it is rather difficult to locate it in such preparations and the conclusions in some cases had to be arrived at after studying a large number of serial sections. Von Ebner's stainable granules and the *sphère chromatophile* are convenient for histochemical studies.

The tests listed in the appendix on page 504 suggest the absence of lipid (even in solid or masked state) from the chromatoid body of spermatocytes and spermatids, the granular satellite of the chromatoid body of the latter, the stainable granules of von Ebner, and the *sphère chromatophile* (figs. 1, A–E; 2, A). The intense basiphilia of these bodies (figs. 2, B–G; 3, A) is

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FIG. 1 (plate). Sections of the testis of *R. norvegicus albinus*; acid haematein (AH) after pyridine extraction.

A, large primary spermatocytes showing in focus AH-positive main chromatoid body (*cb*) adjacent to the nucleus (*n*).

B, early spermatids, duplex chromatoid body indicated by arrows, AH-positive satellite (*s*) in contact with the AH-positive chromatoid body (*cb*) lying on the side of the nucleus (*n*) in one of the spermatids.

C, maturing spermatozoa showing the gradual reduction in the size of the AH-positive chromatoid body (indicated by arrows) situated near the base of the head (*h*).

D, maturing spermatozoa showing AH-positive von Ebner's stainable granules (*sg*), and AH-positive *sphère chromatophile* (*sc*); chromatoid body indicated by an arrow in an early spermatid.

E, irregularly shaped AH-positive *sphère chromatophile* (*sc*) at the front end of the sperm heads (*h*), the one indicated by an arrow has become frothy; some *sphères chromatophiles* being phagocytosed by the Sertoli cell (*st*); chromatoid body in early spermatids indicated by arrows, in one spermatid it is seen inside the manchette (*mt*); satellite (*s*) seen adjacent to the chromatoid body of one of the spermatids.

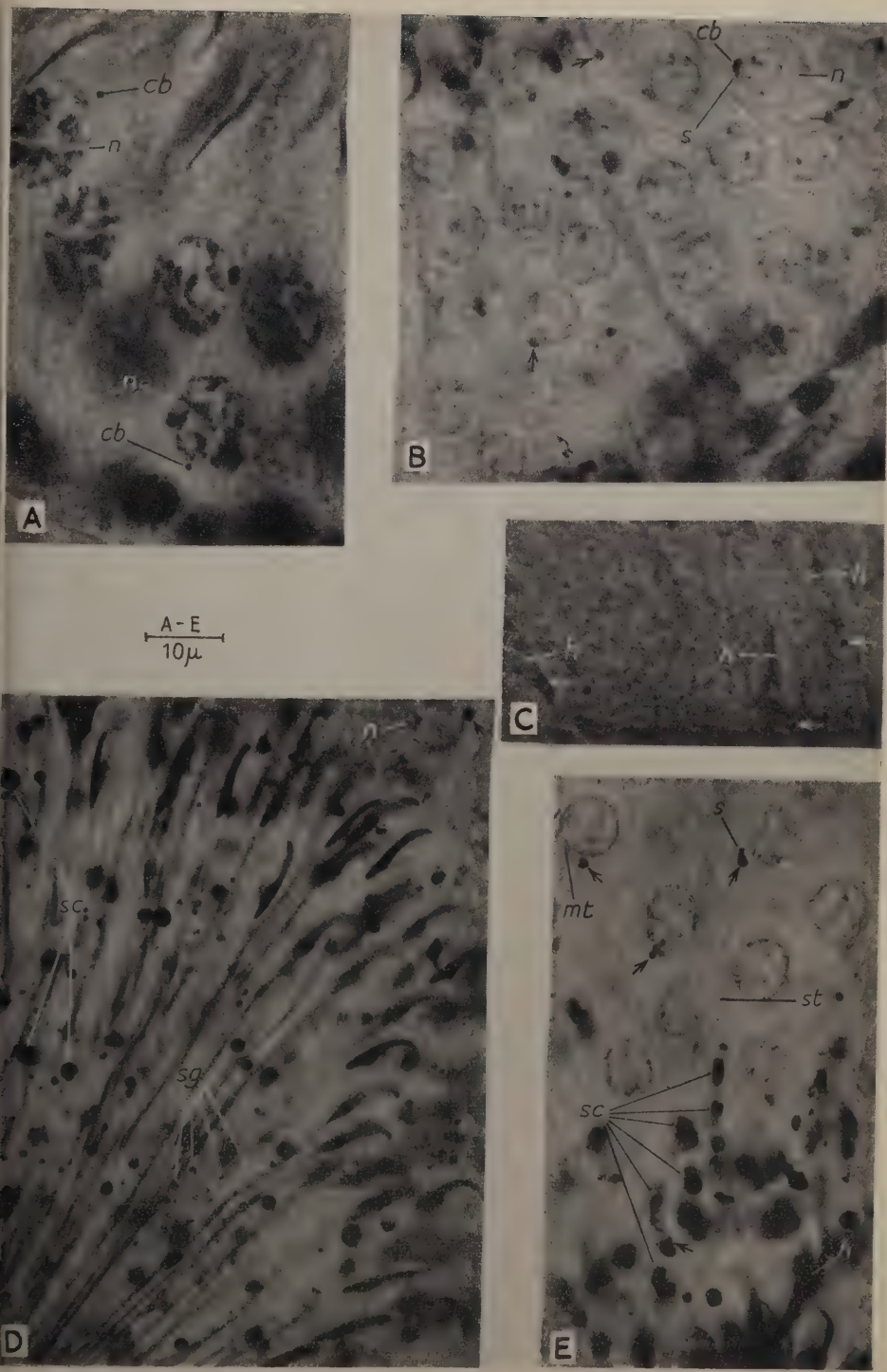

FIG. 1

B. N. SUD

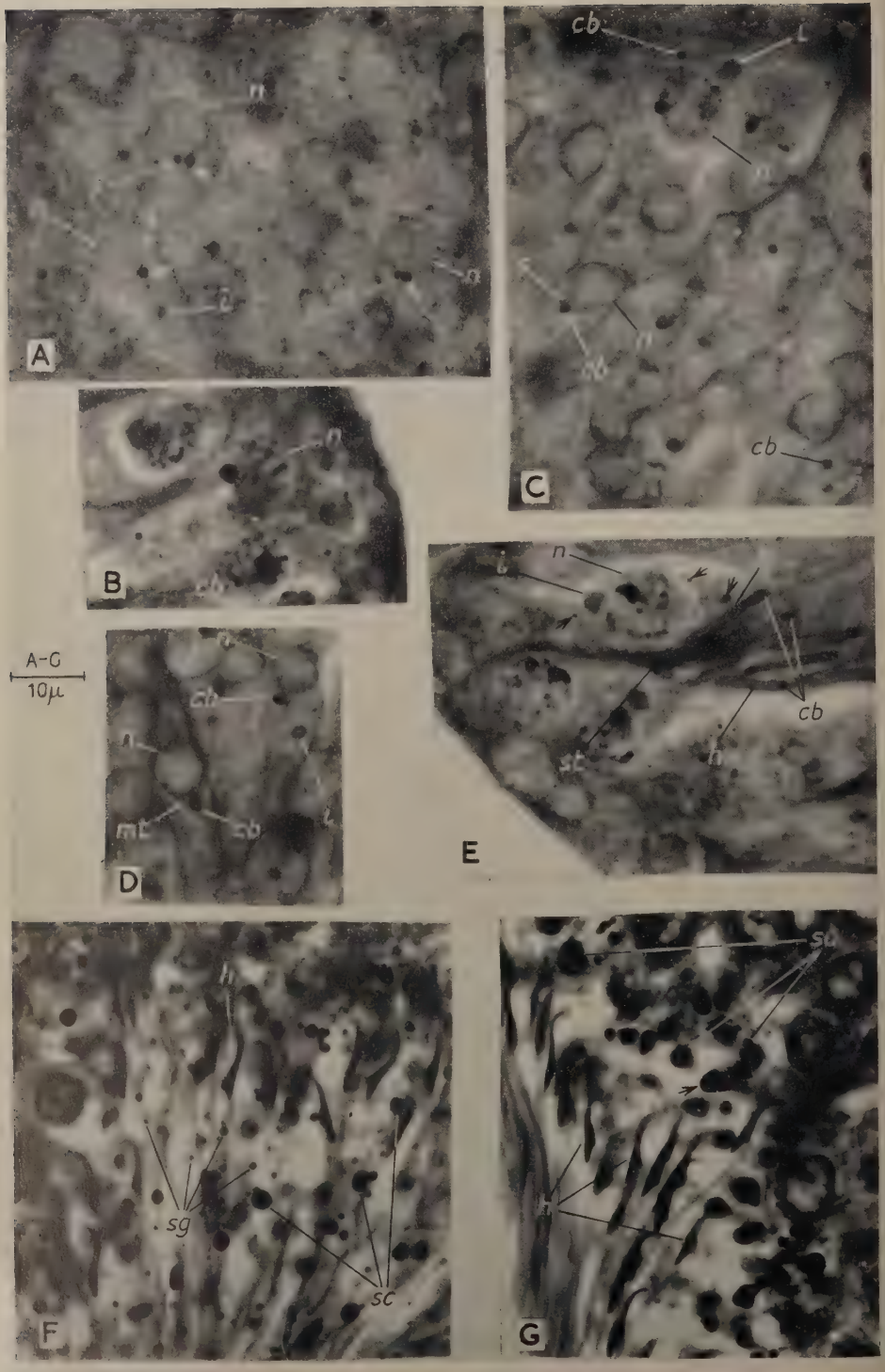

FIG. 2

B. N. SUD

due to RNA. The basiphilia of the satellite may possibly be due to highly polymerized RNA, since it shows special affinity for methyl green in the pyronin / methyl green test, is negative to this test after treatment with ribonuclease, and is Feulgen-negative. It may further be mentioned that the stainable granules, the *sphère chromatophile*, and the satellite stain more readily by some basic dyes (crystal violet and methyl green) than the chromatoid body. This fact suggests that the proportion of RNA in the former is much higher than in the latter. The basiphilia of the structures concerned may also be partly due to acidic groups in protein.

The results of staining with acid dyes (fig. 3, B–F) and the Sakaguchi reaction with and without previous deamination suggest that the chromatoid body, the stainable granules, and the *sphère chromatophile* possess basic proteins (protein-bound arginine) and proteins with basic groups other than amino-groups. Arginine appears to be most abundant in the chromatoid body, less so in stainable granules, and almost absent from the *sphère chromatophile*. Histones appear to be absent from these bodies and arginine therefore presumably occurs as a component of protamines. The satellite lacks basic proteins. There appears to be a small amount of tyrosine in the chromatoid body, the stainable granules, and the *sphère chromatophile*. Tests for alkaline phosphatase, acid phosphatase, and calcium are negative for all the bodies concerned. Thus the histochemical study suggests that the chromatoid body, von Ebner's stainable granules, and Regaud's *sphère chromatophile* are, in general, similar in composition, consisting mainly of RNA and proteins. Further, there is a progressive increase in the RNA component and a corresponding decrease in the arginine component of the chromatoid material (chromatoid body, stainable granules, and *sphère chromatophile*). It also appears that the arginine in these bodies occurs as a component of protamines rather than histones. Protamines are known to replace the histones of chromatin during spermatogenesis.

FIG. 2 (plate). Sections of the testis of *R. norvegicus albinus*.

A, formaldehyde/calcium followed by AH test; early spermatids with AH-positive chromatoid body (indicated by arrows) at the posterior end of the nucleus (*n*); AH-positive satellite (*s*) has detached itself from the chromatoid body.

B, Sanfelice / crystal violet; large primary spermatocyte showing in focus the basiphil main chromatoid body (*cb*) situated away from the nucleus (*n*).

C, Sanfelice / crystal violet; primary spermatocyte (at the top) with basiphil main chromatoid body (*cb*) and faintly stained idiozome (*i*) situated adjacent to the nucleus (*n*); early spermatids showing the chromatoid body (*cb*) near the posterior end of the nucleus (*n*); basiphil satellite (*s*) detached from the chromatoid body seen in one of the spermatids.

D, Sanfelice / crystal violet; the labelled spermatid on the left with the basiphil chromatoid body (*cb*) enclosed by the manchette (*mt*); the labelled spermatid on the right with the faintly stained idiozome (*i*) situated posteriorly to the basiphil chromatoid body (*cb*).

E, Sanfelice / crystal violet; maturing spermatozoa with anterior part of the heads (*h*) embedded in the Sertoli cell (*st*) and reduced basiphil chromatoid body (*cb*) at the posterior end of the nucleus; large primary spermatocyte with the main and two additional basiphil chromatoid bodies (indicated by arrows) and faintly stained idiozome (*i*) lying near the nucleus (*n*).

F, Zenker / basic fuchsine; maturing spermatozoa showing basiphil von Ebner's stainable granules (*sg*) and basiphil *sphère chromatophile* (*sc*).

G, Zenker / basic fuchsine; irregularly shaped basiphil *sphère chromatophile* (*sc*) situated at the front end of the sperm heads (*h*); a frothy *sphère chromatophile* indicated by an arrow.

In the rat, as in many other animals, the spermatid nucleus can be seen to undergo a distinct change in the chemical composition of its chromatin during the late stages of spermateleosis. The following methods were used to study this change.

(*a*) Sections of material fixed in Clarke's (Carnoy's) fluid, when stained with methylene blue, show the chromatin of the spermatid nuclei stained but the nuclei of the spermatozoa are untouched.

(*b*) Sections of material fixed in Tellyesniczky's fluid, postchromed, stained with Ehrlich's haematoxylin and then with safranine (Regaud, 1901*a*) show the chromatin of spermatocytes and spermatids *blue* and that of spermatozoa *red*.

(*c*) Sections of material fixed in Clarke, extracted with hot trichloracetic acid in order to remove DNA, mordanted with aluminium sulphate, stained with haematein, and blued (Baker, technique not yet published) show the chromatin of spermatocytes and spermatids *blue* and that of spermatozoa *brownish*.

(*d*) Sections of the material fixed in Zenker for 3 h, deaminated with van Slyke's reagent, and stained with azocarmine show the chromatin of spermatocytes and spermatids untouched and that of the late spermatids and spermatozoa red.

The distinct change in the chemical composition of chromatin revealed by these tests, the presence of RNA and basic proteins in the chromatoid material, and the gradual decrease in the protein component of the latter suggest that the function of the chromatoid material may be to provide basic proteins for the final maturation of the chromatin of late spermatids.

Discussion

The present investigation has removed all doubts about the presence of the chromatoid body in the primary spermatocyte and spermatid of the rat. It has also proved that the chromatoid body, von Ebner's stainable granules, and

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Fig. 3 (plate). Sections of the testis of *R. norvegicus albinus*.

A, Zenker; pyronin / methyl green; von Ebner's stainable granules (*sg*) and *sphère chromatophile* (*sc*) stained deep red by pyronin; sperm heads (*h*) blue and tails (*t*) red.

B, Sanfelice / acid fuchsine; the labelled cell is a large primary spermatocyte showing the acidophil main chromatoid body (*cb*) near the nucleus (*n*) and a slightly stained idiozome (*i*).

C, Sanfelice / acid fuchsine; early spermatids, the chromatoid body (*cb*) is deeply stained but the idiozome (*i*) is faintly stained.

D, Zenker / azocarmine; the labelled spermatid showing acidophil chromatoid body (*cb*) at the posterior end of the nucleus (*n*).

E, deamination / azocarmine; early spermatids showing acidophil chromatoid body (labelled *cb* or indicated by an arrow), completely negative idiozome (*i*) and chromatin; maturing sperm heads (*h*) are deeply stained.

F, Zenker / azocarmine; acidophil *sphère chromatophile* (*sc*) lying alongside the maturing sperm heads (*h*) or in front of the latter.

G, living cells under phase contrast mounted in a slightly hypertonic protein medium with a refractive index of 1·354; the labelled cell is an early spermatid showing the chromatoid body (*cb*) in contact with its satellite (*s*) lying near the posterior end of the nucleus (*n*), and idiozome (*i*) at the anterior end of the latter showing acrosome-formation.

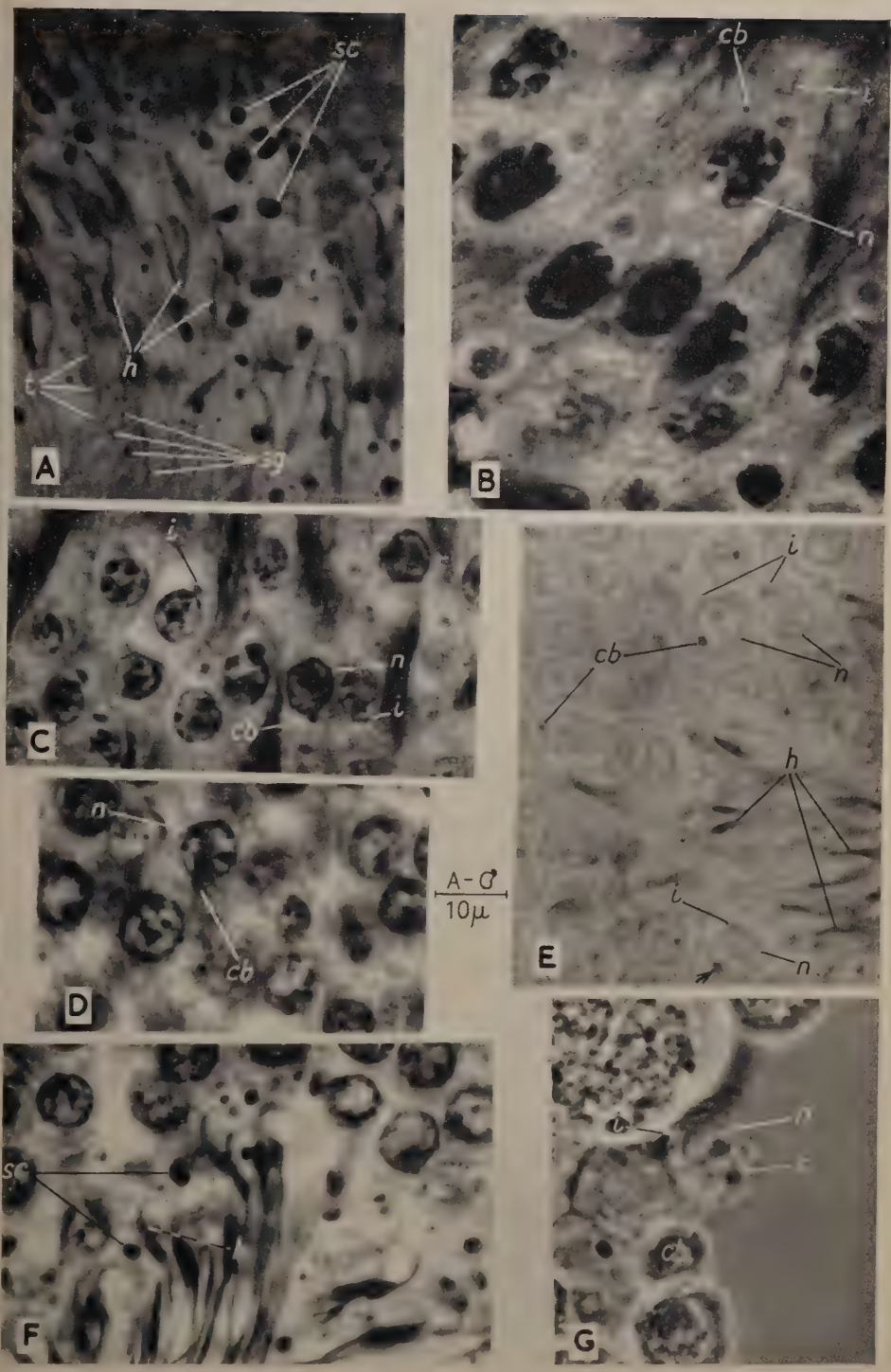

FIG. 3

B. N. SUD

Regaud's *sphère chromatophile* are similar in composition, consisting mainly of RNA and proteins with basic groups. The chromatoid body of the snake (Sud, 1961 *a*, *b*) has a similar chemical composition.

It has been suggested that the chromatoid body of the rat arises from the nucleus (Benda, 1891; Moore, 1893, 1894; Regaud, 1910) or nucleolus (Lenhossék, 1898; von Ebner, 1899; Fischer, 1899; Duesberg, 1908). von Ebner (1888) states that his stainable granules cannot arise from the nucleus since they first appear when the head of the spermatozoon has taken on its final form. I am inclined to think that the chromatoid body and von Ebner's stainable granules originate from the ground cytoplasm, as suggested for the chromatoid body of reptiles (Sud, 1955, 1956, 1957).

As to the fate of the chromatoid body in the rat, a claim has been made that it is absorbed into the nucleus (Moore, 1893, 1894). Other suggestions are that it gradually becomes smaller during the final stages of spermateleosis and then fragments into two or three pieces, which dissolve in turn insensibly into the cytoplasm (Duesberg, 1909), or that it is sloughed off with the residual cytoplasm (Austin and Sapsford, 1951). I believe that during the final stages of spermateleosis the chromatoid body gradually merges into the ground cytoplasm and simultaneously von Ebner's stainable granules arise from the ground cytoplasm in the caudal region and run together to form a single *sphère chromatophile*, which is cast off with the residual body. Some of the residual bodies are phagocytosed by the Sertoli cells while others degenerate in the lumen. According to von Ebner (1888) the stainable granules are derived from the Sertoli cells and they go back into the Sertoli cells. Lacy (1960), however, believes that the RNA of the residual bodies is formed by the germ cells and later enters the Sertoli cells, where it is absorbed or dispersed.

Gresson and Zlotnik (1945) use the term 'accessory bodies' for a cytoplasmic inclusion in spermatogenesis of the rat which they consider to be the same as what others have called chromatoid bodies. According to them the 'accessory bodies' arise from the localized Golgi material. They describe their accessory bodies as argentophil and stainable by iron haematoxylin and acid fuchsine. This supposed homology has already been opposed by myself (Sud, 1961*c*). I have suggested that the 'residual body' described by Gresson and Zlotnik in the spermatid of this species is in fact the chromatoid body. The present investigation supports my earlier opinion.

The granular satellite found in association with the chromatoid body of the spermatid in the present investigation has been shown to differ in its chemical composition from the chromatoid body. The evidence suggests that it consists of highly polymerized RNA. The satellite may be represented by the lamellar arrays that are seen to be associated with the chromatoid body in electron micrographs of the rat spermatid by Swift (1956). It is also just possible that the satellite, after detachment from the chromatoid body, forms the end-knob or basal body of the axial filament, since, like the satellite, the end knob is also stained blue in pyronin / methyl green preparations and both are of approximately the same size.

I take this opportunity for expressing my gratitude to Dr. J. R. Baker, F.R.S., for his kind supervision and helpful discussions, and for translating German and French papers. I am grateful to Professor Sir A. C. Hardy, F.R.S., for providing research facilities in his Department, and to the Vice-Chancellor of the Panjab (India) University for granting me study-leave. I wish to thank Mrs. B. M. Luke and Mr. P. L. Small for their valuable technical help.

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APPENDIX

summary of the histochemistry of the chromatoid body, von Ebner's stainable granules, Regaud's sphère chromatophile, *and the satellite in the rat*

Tests applied					*Results obtained*				
					Chromatoid body				
Test	*Fixation*	*Embedding medium*	*Thickness of sections in μ*	*Reference*	*In spermatocytes*	*In spermatids*	*von Ebner's stainable granules*	*Regaud's* sphère chromatophile	*Satellite*
natural colour					Colourless				
neutral red	living tissue				O	O	O	O	O
Janus green	living tissue				O	O	O	O	O
Sudan black (RT; 60° C)	F/S+PC	G	10	Baker, 1944, 1949	O	O	O	O	O
Sudan black (RT; 60° C)	F/Ca+PC	G	10	Baker, 1946	O	O	O	O	O
Sudan black (RT; 60° C)	WB+PE	G	10	Baker, 1946	O	O	O	O	O
Sudan black (RT; 60° C)	Aoy+PC	G	10	Baker (unpublished)	O	O	O	O	O
Sudan black (RT; 60° C)	Cr/F+PC	G	10	Lewitsky, 1931	O	O	O	O	O
Sudan black (RT; 60° C)	Flm+PC	G	10	Bradbury and Clayton, 1958	O	O	O	O	O
Sudan black (RT; 60° C)	LS	G	10	Baker, 1956*a*	O	O	O	O	O
acid haematein	F/Ca+PC	G	10	Baker, 1946	O	+++or+++ and + parts	O	O	O
acid haematein	WB+PE	G	10	Baker, 1946	+++	+++ or +++ and+parts	+++	+++	+++
acid haematein	Aoy+PC	G	10	Baker (unpublished)	O	+++ or +++ and+parts	O	O	+++
acid haematein	Cr/F+PC	G	10	Chayen and others 1959	+++	+++ or +++ and O parts	O	O	+++
acid haematein	Flm+ PC	G	10	Bradbury and Clayton, 1958	O	+++ or +++ and O parts	O	O	+++
Liebermann	F/Ca; F/Ca+ PC	G	15	Lison, 1953	O	O	O	O	O
silver impregnation	Aoy	P; G	6; 2	Aoyama, 1929	O	O	O	O	–
Weigl (Mann–Kopsch)	Mann PO	P	2	Weigl, 1910	O	O	O	O	–
Rawitz	Flm	P	6	Baker, 1959; Przełęcka, 959	O	O	O	O	–

APPENDIX (*cont.*)

Tests applied					*Results obtained*				
					Chromatoid body				
Test	*Fixation*	*Embedding medium*	*Thickness of sections in μ*	*Reference*	*In spermatocytes*	*In spermatids*	*von Ebner's stainable granules*	*Regaud's* sphère chromatophile	*Satellite*
toluidine blue for metachromasy	Z	P	10	Baker (unpublished)	O	O	O	O	O
thionine for metachromasy	Z; Cl	P	10	Baker (unpublished)	O	O	++	++	–
periodic acid / Schiff	Z	P	8	McManus, 1948; Pearse, 1954	O to +	O to +	O to +	O to +	O
Feulgen	Z; Cl	P	6; 8	Feulgen and Rossenbeck, 1924	O	O	O	O	O
pyronin / methyl green	Z 3 h	P	7; 16	Jordan and Baker, 1955	+++ (red)	+++ (red)	+++ (red)	+++ (red)	+++ (blue)
P/MG after trichloracetic acid	Z 3 h	P	7; 16	Pearse, 1954; Jordan and Baker, 1955	++ (red)	++ (red)	+ to ++ (red)	+ to ++ (red)	++ (red)
P/MG after ribonuclease	Z 3 h	P	7; 16	Bradbury, 1956	O	O	O	O	O
basic fuchsine (0·5% aq.; RT) for basiphilia	Z 3 h	P	7	—	+++	+++	+++	+++	+++
safranine (0·5% aq.; RT) for basiphilia	Z 3 h; LS	P	6	—	+++	+++	+++	+++	+++
safranine (0·5% aq.; RT) after trichloracetic acid	Z 3 h	P	6	Pearse, 1954	O	O	O	O	O
Newton's crystal violet	Z 3 h; LS; Cl	P	6	Baker and Jordan, 1953	O	O	O to +	O to +	+++
Newton's crystal violet (staining at 50° C)	Z 3 h	P	6	Baker and Jordan, 1953	O	O	+++	+++	+++
Newton's crystal violet	S	P	16	Baker and Jordan, 1953	+++	+++	+++	+++	+++
Newton's crystal violet after trichloracetic acid	S	P	16	Baker and Jordan, 1953; Pearce, 1954	O	O	O	O	O
Cresyl violet as used for Nissl bodies	Z 3 h	P	6; 16	Fernstrom, 1958	++	++	+++	+++	–
methyl green (1% aq.; RT) for basiphilia	Z 3 h	P	7	—	O	O	+++	+++	–
methyl green (1% aq; 50° C) for basiphilia	Z 3 h	P	7	—	+++	+++	+++	+++	–
iron haematoxylin	Z 3 h	P	7	—	+++	+++	+++	+++	+++
Ehrlich's haematoxylin / safranine (Zwaardemaker's)	T 24 h	P	6	Regaud, 1901*a*	+	+	+++	+++	–

APPENDIX (*cont.*)

Tests applied					*Results obtained*				
					Chromatoid body				
Test	*Fixation*	*Embedding medium*	*Thickness of sections in μ*	*Reference*	*In spermatocytes*	*In spermatids*	*von Ebner's stainable granules*	*Regaud's* sphère chromatophile	*Satellite*
acid fuchsine (1% aq.; RT) for acidophilia	S	P	6	—	+++	+++	+++	+++	O
eosin (1% aq.; RT) for acidophilia	Z 3 h	P	7	—	—	+++	+++	+++	O
xylidine red (0·1% in 1% acetic acid; RT)	Z 3 h	P	7	—	—	++	++	++	O
azocarmine (2% aq., acidified; 50° C)	Z 3 h	P	7	Gatenby and Beams, 1950	+++	+++	+++	+++	O
deamination by van Slyke reagent / azocarmine (2% aq., acidified; 50° C)	Z 3 h	P	7	Monné and Slautterback, 1950	+++	+++	+++	+++	O
Sakaguchi	Z	P	16	Baker, 1947	++	++	+	O to +	O
deamination by van Slyke reagent / Sakaguchi	Z	P	16	Monné and Slautterback, 1950, Baker, 1947	O	O	O	O	O
Hg/nitrite	F/S; Alc	C, PCP	26	Baker, 1956*b*	O to +	+	O to +	+	O
coupled tetrazonium	F; Alc	P, PCP	6, 10	Danielli, 1947, 1950; Pearse, 1954	—	+	+	+	O
alkaline fast green method	F	P, PCP	6, 10	Alfert and Geschwind, 1953	—	O	O	O	O
Gomori's for alkaline phosphatase	Alc/Acet	P	8	Gomori, 1952	O	O	O	O	O
lead method for acid phosphatase	Acet	P	8	Gomori, 1952	O	O	O	O	O
cobalt method for calcium	F/M/P	P	10	As in alkaline phosphatase test used by Danielli, 1946	O	O	O	O	O

Key. Alc = alcohol; Acet = acetone; Alc/Acet = absolute alcohol and acetone in equal volumes; Aoy = Aoyama's fluid; aq. = aqueous; C = collodion; Cl = Clarke's (Carnoy's) fluid; Cr/F = chromium trioxide/formaldehyde; F = formaldehyde; F/Ca = formaldehyde/calcium; Flm = Flemming's fluid; F/M/P = formaldehyde/methanol/pyridine; F/S = formaldehyde/saline; G = gelatine; LS = Lewitsky-saline; P = paraffin; PC = post-chromed; PCP = Peterfi's celloidin-paraffin method; PO = postosmicated; RT = room temperature; S = Sanfelice's fluid; T = Tellyesniczky's fluid; WB+PE = weak Bouin+pyridine extraction; Z = Zenker's fluid; 3 h = 3 hours; +++ = strong reaction; ++ = moderate reaction; + = weak reaction; O = no reaction; — = no observation.

A Study of the so-called 'Chloride-Secretory' Cells of the Gills of Teleosts

By T. VICKERS

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With one plate (fig. 1)

Summary

The histology of the gills of a normally freshwater teleost, the guppy (*Lebistes reticulatus*), has been studied after the fish had been subjected to various concentrations of sodium chloride. Cells similar to those identified by previous workers as 'chloride-secretory cells' develop in response to adaptation to hypertonic salt solutions, and the number and character of the cells can be related to the salt load and to the degree of adaptation of the fish to it. The cytology of these cells has been shown to be consistent with their supposed function. In fish which are not fully adapted to the salt load, these cells show signs of the formation of mucous materials, and under extreme conditions they may undergo transformation into goblet cells. This may provide an explanation of the failure of Bevelander (1935, 1936) to find any specialized cells other than mucous cells on the teleost gill.

Introduction

THE work of Schlieper (1935), Keys (1931, 1933), and Bateman and Keys (1932) has shown that extra-renal mechanisms may play a substantial role in the ionic balance of teleosts. A marine teleost is liable to dehydration because, despite the inability of the kidney to form a urine significantly hypertonic to the blood, it maintains its body-fluids considerably hypotonic to sea-water. This process depends on the fish drinking sea-water and absorbing the water and monovalent ions from the intestine; the excess ions, principally sodium and chloride, are then excreted by a mechanism located in the anterior part of the fish. Keys's experiments showed that the gills were active in this respect and that the osmotic work performed was very considerable, accounting in the eel for as much as a third of the total oxygen consumption. The cells responsible for this ionic transfer must, therefore, be highly specialized for the performance of osmotic work and should be identifiable histologically.

Keys and Willmer (1932), in a study of the gills of the eel, described cells which, they suggested, might be responsible for the ion transport and which they called 'chloride-secretory cells'. It should be pointed out that Keys's analytical methods enabled him to estimate only chloride, and the question of which ion is actively transported, i.e. moved against an electrochemical gradient, is left undecided. The term 'chloride-secretory cell' will be used here for convenience, without any implication of an active transport of chloride (sodium may well be the ion which is actively transported), and indeed without any prejudice at all as to the cell's function.

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The cells were situated at the bases of the gill platelets, especially near the afferent branchial vessels, and were described as large, spherical or ovoid cells with eosinophil, granular cytoplasm.

In a study of the branchial tissues of various species of fish, Bevelander (1935, 1936) denied the existence in the gill epithelium of any specialized cells other than mucous cells, and suggested that the entire gill epithelium was responsible for the salt transport. He was supported in this view, although for no clearly stated reasons, by Smith (1951) and Krogh (1939). This disagreement with Keys and Willmer is of fundamental importance, since many of Bevelander's illustrations show cells which are similar to those which Keys and Willmer (and most later workers) identify as chloride-secretory cells. Bevelander relied mainly on metachromatic staining of material in the cells. He used thionine as an indicator of the presence of mucin. More recently, however, doubt has been cast on the relevance and reliability of the metachromatic staining property of thionine, which has been ascribed to the presence of a red impurity in the dye (Pearse, 1960). Bevelander also pointed out that the cells were not restricted to the gills, but were of general occurrence in the buccal cavity. The cells were found to stain vitally with the Janus green and neutral red; that observation is at least as consistent with the identification of the cells as chloride-secretory cells as with their being mucous cells.

Pettengill and Copeland (1948) studied the histology of the gills in an estuarine fish, *Fundulus heteroclitus*, under various conditions of salt loading. They agreed with Keys and Willmer's suggestion and drew attention to the presence at the free border of the cells of a vesicle, which they found to contain chloride when the animal came from salt water; this is perhaps not remarkable, since the vesicles often appeared to open to the outside. They also demonstrated the presence of alkaline phosphatases, both enzyme and vesicle undergoing changes associated with the movements of the fish between salt and fresh water. They also noted a resemblance between the cells of the pseudobranch (a modified gill on the first gill arch) and the chloride-secretory cells.

Burns and Copeland (1950) have further characterized the cells in *Fundulus*. They found the cells to be strongly osmiophil and to be distributed, more widely than hitherto suspected, in the buccal cavity generally; this finding obviously constitutes a partial answer to Bevelander. They made the interesting point that the number of the cells at any point in the buccal cavity or on the gills was directly correlated with the degree of vascularization at that point; this is consistent with the suggested role of movement of ions from the blood to the external environment.

Getman (1950) studied the cells ofeels from freshwater and sea-water. He confirmed the osmiophilia found by Burns and Copeland and noted a pit at the free border of the cells, which he regarded as characteristic of adaptation to salt water and as equivalent to the vesicles described by Pettengill and Copeland in *Fundulus*.

Liu (1942) showed that the normally freshwater teleost, *Macropodus*

opercularis, could be adapted to life in hypertonic salt solution, and that only then were chloride-secretory cells present on the gills. Huntsman and Hoar (1939) found that the cells developed in salmon when entering the sea at the parr-smolt transition; these results support the suggestion of Keys and Willmer.

Despite the considerable number of studies of these cells, it seems that they will bear still further investigation, since Bevelander's arguments have never been answered or even explicitly discussed. The most promising approach seemed to be that previously adopted by Liu: to relate changes in the structure of the gills of a normally freshwater fish to the development of the capacity for salt excretion.

Materials and Methods

Guppies (*Lebistes reticulatus*) were used for most of the experiments. The original stock was purchased locally, and thereafter was maintained by breeding; 120 fish were used, of which 80 provided material for histology.

The main stock of guppies was kept in tanks containing about 25 l. of water held thermostatically at 26° C. Experimental animals were kept at the same temperature; either in jars holding 600 ml, or in tanks holding 5 l. In all cases the water was artificially aerated by a small mechanical pump.

In most experiments involving adaptation to increasing salt concentrations, the salt was either added at appropriate intervals as a 2 M solution or a continuous drip of a concentrated solution was arranged. Slow adaptation was most conveniently achieved by controlled evaporation of the water. The concentration of the media was checked by titration of samples against standard silver nitrate.

The fish were killed with 0·5% urethane, and the entire animal was fixed after opening the abdominal cavity to allow penetration of the fixture. The best general fixative was Zenker's fluid modified by replacing the acetic acid of the original formula with the same volume of formic acid to decalcify the material. Some fish were fixed in Bouin's fluid, and in this case also the best results were obtained by replacing the acetic acid of the original formula with formic acid. For cytological study, the fish were fixed in Helly's fluid, postchromed in 3% potassium bichromate, and then treated, after thorough washing in running water, with one of the decalcifying fixatives. Fixation in ice-cold acetone was used for studies of enzyme distribution, but, in general, any fixative which did not itself decalcify or which was not followed by a decalcifying agent gave an almost impossible texture for sectioning. Studies of esterase distribution were made on whole gill bars fixed for short periods in 10% neutral formalin. I have not found it possible to prepare satisfactory frozen sections of gill tissue, even after gelatine-embedding.

After fixation, the tissues were dehydrated, cleared, embedded in paraffin, and sectioned, usually at 3 or 4 μ, and the sections mounted serially. A disadvantage of the guppy as histological material is that the cells are extremely

small, and very thin sections are necessary for anything more ambitious than micro-anatomy.

Observations

The ability of the guppy to adapt to sudden changes of salinity was tested by the transfer of fish to solutions of sodium chloride covering the range of concentration of 0·15 M to 0·5 M. All the fish except those in the lowest concentration died within a period of 24 h; in 0·5 M sodium chloride all were dead within 2 h. The ability of the gills of the normal guppy to excrete salt is therefore small; this point is obviously crucially important.

In further experiments, fish were transferred to 0·15 M sodium chloride and then gradually adapted to higher concentrations. If the molar concentration of the solution was increased at the rate of 0·03 each day, either by addition of a concentrated solution twice daily or by continuous addition with a drip-feed system, the fish adapted, with a mortality of about 50%, up to about 0·35 m sodium chloride, which seemed to represent the upper limit for adaptation. With diluted sea-water, however, adaptation could be continued well beyond this level at a rate of increase in molarity of 0·25 each day.

The reason for the difference between sea-water and sodium chloride solutions presumably lies in the other ions present in the former; calcium and magnesium are perhaps the most likely to be important.

The state of adaptation could be judged by a pronounced darkening of fish, apparently as a result of melanophore activity, when they were failing to adapt; the darkening disappeared when the fish were returned to a slightly lower concentration, and adaptation could then continue at a lower rate.

In this way, fish from fresh water could be adapted to full-strength sea-water in about 2 weeks.

Above about 0·5 M sodium chloride (i.e. in sea-water with extra sodium chloride), adaptations to additions of salt were rather precarious, but, if the increase in concentration was affected by the evaporation of the sea-water, adaptation was possible up to a limit of about 1·2 M sodium chloride; in this way all the ionic concentrations were, of course, proportionately increased.

The fish were normally kept in pairs or threes, and, by choosing animals for histological investigation according to their own and their companions' reactions, it was possible to obtain material representing various states of adaptation and of failure to adapt.

To produce the characteristics of complete adaptation, a group of fish adapted to full-strength sea-water was kept for several weeks with the concentration maintained constant; another group was kept in 0·125 M sodium chloride for a week to test the effects of an approximately isotonic medium.

Comparison of the gills of fish adapting to salt with those of controls (fig. 1, A) showed that, in the former (fig. 1, B), cells were present similar in description to those called 'chloride-secretory cells' by Keys and Willmer. They were apparently represented in the controls only by small cells with no conspicuous staining properties. They were found, as described by Keys and Willmer, at

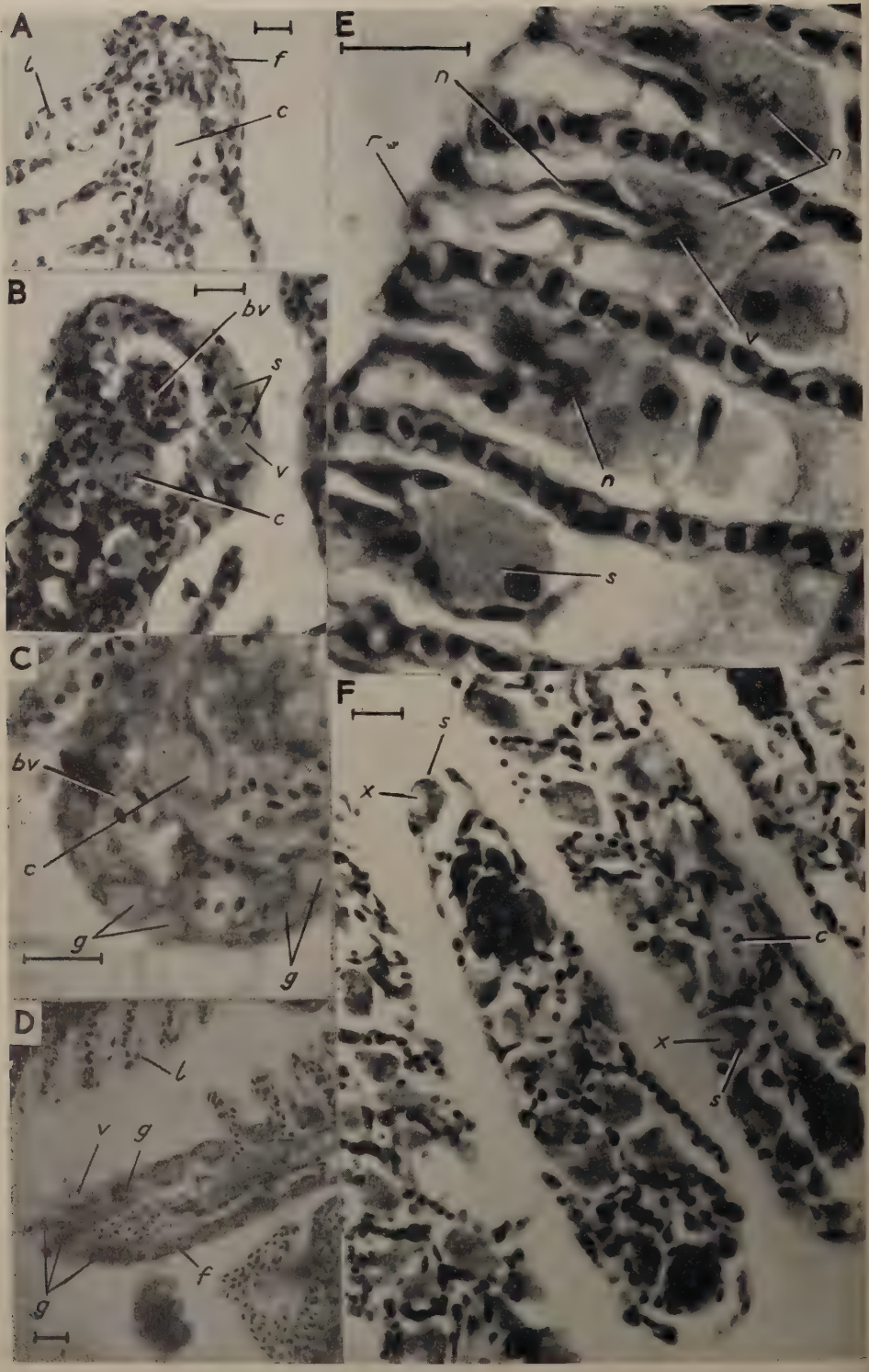

FIG. 1

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the bases of the gill lamellae and along the edge of the gill filaments, adjacent to the afferent branchial artery. As compared with the other cells in the guppy the cells were large, with granular, eosinophil, siderophil, and (even in paraffin sections) sudanophil cytoplasm. The sudanophilia was presumably due to phospholipid. The granules reacted strongly to both the Barrnett (1953) and Bennett (1951) methods for sulphydryl groups, and were presumably mitochondria; this identification has been confirmed by electron microscopy (Vickers: unpublished observation), and probably explains the uptake of Janus green noted by Bevelander.

At the free border of the cell there was usually a vacuole or vesicle, sometimes opening to the surface. This was bounded by a rim of strongly basiphil material also staining strongly with the periodic acid / Schiff (PAS) reaction and therefore presumably containing an acid mucopolysaccharide; the basiphilia was not removed by treatment with salivary ribonuclease (Bradbury, 1956); there was, however, no metachromasia after staining with toluidine blue.

Especially at the edge of the filaments, these cells could be seen to lie beneath the epithelioid covering of the gills and to be adherent to it; the only apparent interruption in the continuity of this sheath was where the vesicle of the chloride-secretory cells opened to the exterior through it; I have confirmed this observation with the electron microscope.

The cells gave no reaction to the Gomori (1941) and Seligman (Pearse, 1960) methods for alkaline phosphatase, although other structures in the sections showed intense activity, the cells of the respiratory epithelium itself being most conspicuous.

Whole gill arches reacted strongly to the diazonium method for esterase (Pearse, 1960), and the strongest activity could be seen to be between the lamellae at the sites occupied by the supposed chloride-secretory cells. The

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FIG. 1 (plate). Gills of *Lebistes reticulatus*. The scales represent 10 μ.

A, control fish. Stained with haematoxylin and eosin. The end of a gill filament is shown with 5 lamellae. There are no cells which show any obvious differentiation of the chloride-secretory pattern.

B, fully-adapted to sea-water. Stained with haematoxylin and eosin. Cells of the chloride-secretory type are present; only one shows a vesicle, and that small. The afferent branchial vessel is visible.

C, failure to adapt to salt. Stained with Azan. Four cells show the mucous transformation. Note that there are no cells recognizably of the chloride-secretory type.

D, failure to adapt to salt. PAS technique. Some cells show a positive reaction for mucus; one other has a very large vesicle. There are small chloride-secretory cells between the lamellae.

E, fish from 1·2 M NaCl. PAS technique. This shows the vesicle opening through the respiratory epithelium and the PAS-positive network around the vesicle. The blood-vessels within the lamellae are clearly shown.

F, gills from the same fish as E; stained with iron haematoxylin. The chloride-secretory cells are packed with granules (mitochondria) except in the area (*x*) corresponding to the network in E.

bv, blood-vessel; *c*, cartilage; *f*, gill filament; *g*, chloride-secretory cell in mucous transformation; *l*, gill lamella; *n*, network around the vesicle; *r*, respiratory epithelium; *s*, chloride-secretory cell in normal form; *v*, vesicle; *x*, clear area around the vesicle.

reaction was greatly reduced by pre-treatment of the tissue with 10^{-4} M eserine. More precise definition of the esterase has not been possible; it was not present in gill tissues fixed with acetone, although, again, other structures such as pronephric tubules give a strong reaction in the same sections.

In the fish chronically adapted to sea-water, the cells were even more strongly eosinophil and granular, the sudanophilia and siderophilia were more intense, the vesicle smaller, and the conspicuous staining of the rim absent; cells of the chloride-secretory type were also to be found in the lining of the buccal cavity and on the inner surface of the operculum.

In young fish, however, chloride-secretory cells were normally present in the control animals, although smaller and with generally weaker staining reactions than in adults adapting to salt. So far as I have been able to determine, these cells regressed to the normal adult control picture at about the same age as the secondary sex characteristics developed. The reactions of young fish to salt loads were the same as in the adult animals, except that the cells did not show any appreciable change until the fish had reached 0·15 M sodium chloride. It is clear that the cells which resembled the chloride-secretory cell in the young control fish were the same ones later present in hypertonic saline; there were not two distinct populations of cells.

The presence of these cells when presumably no salt excretion was necessary is a point against their identification with that function, but one possible explanation is that the same cells might be responsible not only for excretion of salt but also for its absorption. In the freshwater teleost, although the kidney conserves salt with great efficiency, the gills absorb ions from the environment (Krogh, 1939). It may be that the kidney of the young fish is relatively ineffective, and that more activity is thus required of the gills; certainly, the kidney is still increasing in size in a very marked way up to the time when the gill cells regress. However, in the immature fish kept in what must have been almost isotonic saline and in which, if the above explanation were correct, the cells might have been expected to regress to the adult picture, there was, in fact, no obvious change in the cells. For the time being this must be held as evidence against these cells being responsible for ionic regulation. It is, however, known that considerable changes in the pattern of osmoregulation may take place at puberty in fish (Black, 1957).

In fish adapted to salt concentrations lower than that of sea-water, the cells were smaller, but the number in the gills was roughly the same; apparently the precursor cells in the gills are all of approximately the same responsiveness to the stimulus of salt load, whereas the precursors of the chloride-secretory cells elsewhere have higher thresholds, and more and more of the latter cells differentiate as the strength of the stimulus increases. This process could be well seen in fish exposed to very high concentrations of salt: the spread of the chloride-secretory tissue into the buccal cavity was then sometimes very marked, and structures of a glandular nature could often be seen in the floor of the buccal cavity, with groups of chloride-secretory cells at their bases. The cells on occasion spread farther, on to the outer surface of the body, occupying

sites in pockets under the scales, where normally only apparently undifferentiated cells and an occasional goblet cell were to be found. This potential distribution of chloride-secretory cells on the body-surface seems to be restricted to the ventral and lateral surfaces and not to extend backwards beyond the pectoral fins.

In fish which were failing to adapt to the salt load (fig. 1, C–F) and which were showing either the darkening previously described or more overt symptoms of distress, the differences already described, between fish which were adapting and those which had adapted, were represented in a more extreme form; the eosinophilia, siderophilia, and sudanophilia of the cells were much weaker, the vesicle was larger, and its rim more prominent.

Fish in this state usually died within about 24 h unless the salt load was reduced, but in a few cases recovery was spontaneous, and some of these showed what appeared to be transformation of chloride-secretory cells into mucous cells; complete graded series could be traced, from cells easily recognizable as chloride-secretory cells, through cells more characteristic of failure to adapt, with an enlarged vesicle and reduced mitochondrial staining, cells in which the vesicle had begun to displace the nucleus into the base of the cell and in which the rim of the vesicle was intensely basiphil, to cells, indistinguishable from goblet cells, with the vesicle full of basiphil material giving a positive PAS reaction, and, in some cases, showing strong metachromasia with toluidine blue. This is, then, apparently an example of a cell undergoing transformation from one mode of differentiation into another.

This interpretation rests on the following argument. The sequence of changes that has just been described was found most commonly at the edges and, more especially, the tips of the gill filaments; few changes were found in the cells between the lamellae. Since, at the sites where the change occurred, almost every cell normally responded to the stimulus of salt loading by forming a cell of the chloride-secretory type, and since this may reasonably be assumed to have occurred before the fish began to fail to adapt, there must have been few undifferentiated cells remaining which could develop afresh into goblet cells. Since also mitoses, which would provide new undifferentiated cells, were very rare in the gill epithelium, and since there were normally no goblet cells in the gills of the guppy, it is most probable that the changes did in fact occur in the differentiated chloride-secretory cell.

The rarity of this apparent transformation should be emphasized; but, in each of the three cases where it was found, there was a complete gradation of intermediate forms, and some significance must surely attach to the fact that each of these fish had the same history of recovery from the symptoms normally associated with failure to adapt, whereas goblet cells, or any of the supposedly transitional forms, were not found in any of the other fishes examined.

Clearly, the formation of a layer of mucus over the gills might be advantageous in reducing their permeability, and hence in decreasing the osmotic loss of water (see van Oosten, 1957), but, although the transformation of chloride-secretory cells might theoretically be valuable in this way and presumably

explains the recovery of these fish, the conditions under which the change took place seem to be so rarely fulfilled that it can hardly be of real functional significance so far as the whole animal is concerned.

However, the chloride-secretory cells in all animals in the process of adaptation to increases in salt load showed the rim of mucous material round the vesicle; and the formation of small quantities of mucus may be a normal feature of the cell, possibly playing a part in the determination of the permeability of the active areas of membrane or even being a biochemical concommitant of ion transport (Willmer, 1960). It may be that, if the salt load exceeds the capacity of the cell, more mucus is produced in an attempt to assist the active mechanisms, until, in extreme cases, the formation of mucus forms the major histologically recognizable differentiation of the cell.

At first sight this appears to be an example of a transformation of one cell type into another, a phenomenon which is usually said to be rare or even non-existent, but the change in the chloride-secretory cell seems to be more appropriately regarded as a shift in the balance between two cell activities which are normally simultaneously present. The presence of mucus in cells concerned with ion movements is not unusual; what is surprising is the extent to which that synthetic activity can become the predominant characteristic of the cell.

The way in which the organelles of the chloride-secretory cell are involved in this change is obviously of some interest, but elucidation of this must await electron microscope studies of the phenomenon.

The extreme smallness of the cells of the guppy makes cytological study difficult, but this disadvantage was partly overcome by the use of fish adapted to solutions more concentrated than sea-water, when the chloride-secretory cells might be as large as 15 to 20 μ in diameter. In these fish, a space could be seen, surrounding the vesicle, free from mitochondria; and the stainable rim of the vesicle could be resolved as a twisted network, lying within this space, which stained with aldehyde fuchsin and with iron haematoxylin. In some fish, usually those in which the eosinophilia of the cells was least marked, the network was seen to stain metachromatically with toluidine blue, and, although the further stages of the transformation were never seen in this group of fish, this may be regarded as a manifestation of the mucous change already described.

In the cells in the wall of the buccal cavity, where there was usually considerable differential shrinkage of the chloride-secretory cells within the presumably rigid, keratinized epithelium, the network could often be seen to be stretched out so that its strands were shown to radiate from the vesicle into the granular area, and the presence of beadings were seen along the length of the strands.

At first I believed these threads or strands to be, either alone or in association with the vesicle, the equivalent in the chloride-secretory cell of the Golgi apparatus; attempts to confirm this by the use of osmium tetroxide as specified by Baker (1950) or by silver techniques, for example that of Elftmann (1953), failed because, in both cases, much was impregnated which could hardly be

Golgi apparatus. Using the electron microscope, I have found nothing to resemble the structure now supposed by electron microscopists (e.g. Sjöstrand, 1956) to represent the Golgi apparatus, and the threads radiating from the vesicle are seen to represent groups of endoplasmic tubules apparently draining into the vesicle. The similar failure of Hally (1960) to find, in the oxyntic cell of the stomach, structures corresponding to the Golgi apparatus of electron microscopists may be of relevance, in view of the similar appearance of the two types of cells, the presence in both of an intracellular system opening on to a surface, and the supposed association of each with ion movement.

Some other species were also tested for a relationship between changes in gill structure and the development of ability to excrete salt; goldfish (*Carassius auratus*) would tolerate transfer direct to 0·15 M sodium chloride and could then be gradually adapted to concentrations up to about 0·25 M. It is known (Krogh, 1939) that under these conditions in this species the blood concentration of salt rises parallel with the external concentration; there is no active regulation of the blood sodium chloride when the fish is in 'hypertonic' salt solutions.

The gills of this species showed no reaction to salt loading; the picture was consistently similar to that of the freshwater fish, and there were no cells identifiable as chloride-secretory cells.

The minnow (*Phoxinus phoxinus*) was exactly the same as the goldfish, showing no cytological signs of adaptation.

In both these species the limited powers of physiological adaptation, and those apparently shown by all the tissues rather than representing the development of an excretory mechanism, may be taken, with the inability of the gills to form cells of the chloride-secretory type, as favouring the identification of that cell type with its suggested function.

Discussion

The histological techniques used in the work described necessarily give only indirect evidence as to the functions of the cells, but, within these limits, the results support Keys and Willmer in their identification of the cells responsible for extra-renal salt regulation.

Keys's work indicated that ion transport must account for a considerable part of a marine teleost's energy utilization, and the cells described, rich in sulphydryl groups, phospholipids, and mitochondria, satisfy the primary requirement of being well equipped for such work. Similarly, the cells are well situated to carry out the function ascribed to them, with a profuse blood-supply on one side and in close contact, although perhaps not as close as was previously assumed, with the external medium on the other.

The salt load to which a fish was exposed and the degree of adaptation of the fish to that load were closely associable both with the structure of the individual cell and with the number and distribution of the cells.

The picture of the enzymic constitution of the cells is not so clear-cut. My failure to find any appreciable phosphatase activity in the cells contrasts with

the report of Pettengill and Copeland, who claim to find by the use of the Gomori technique an enzyme or enzymes capable of hydrolysing many phosphate esters in the presumably equivalent cells in *Fundulus*. I used, in addition to the Gomori technique, that of Seligman (Pearse, 1960), which appears in my experience to be the more reliable. Without careful controls, the Gomori technique may easily give false localizations, and spurious reactions may result, for example, from failure to remove non-specifically bound cobalt after the conversion of calcium phosphate to cobalt phosphate. Although the chloride-secretory cells were inactive, other sites in the same sections showed activity; the implication is that the enzyme, if present in significant amounts, would have been demonstrated in my preparations. Other studies (Gomori, 1941; Browne, Pitts, and Pitts, 1950) of alkaline phosphatase distribution in fish and mammals have, however, shown inexplicable species differences, and no great significance can yet be attached to such anomalies.

The only other enzyme which I have been able to demonstrate is the rather ill-defined esterase. Without further work, consideration of its intracellular localization and its exact nature would be unjustified.

One possible explanation of Bevelander's denial that there are any specialized cells in the gills, other than mucous cells, has already been proposed on the basis of the pseudo-metachromasia shown by thionine. Alternatively, in view of the mucous transformation of which the chloride-secretory cell is apparently capable, and its (slight) content of mucoid material except when the fish is fully adapted to the environment, his fish may have been in conditions such as to cause mucus to be produced. Salt excretion is a very labile function in fish (see Black, 1957), and if this lability extends to the cells responsible for salt excretion, it may be that the larger species studied by Bevelander are less reliable material than fish like guppies, which seem to be very readily adaptable to aquarium life.

I agree with him (and with Burns and Copeland) that these cells, whatever their identity, are not restricted to the gills but may occur far more widely. Their distribution, however, is only relevant to a discussion of their function in so far as their presence or absence on the gills is concerned: Keys's experiments did not eliminate the possibility of contributions to the extra-renal salt excretion by tissues other than the gills.

A more sophisticated argument has recently been advanced by Parry, Holliday, and Blaxter (1959); they point out that, since the pseudobranch is apparently composed of chloride-secretory cells, some association between that gland and salt metabolism should be demonstrable if the similar cells on the gills do in fact excrete salt. They have, however, failed to find any evidence for such an association, but, instead, have found that in some way, possibly as an endocrine gland, the pseudobranch affects the pigmentation of the skin. They feel that this casts doubt on the function ascribed to the chloride-secretory cell.

I have studied the pseudobranch in a number of my fish, and I agree that there is a very close resemblance between its cells and the chloride-secretory

cells of the gills. This resemblance extends to the structure as shown in the electron microscope studies of the pseudobranch (Copeland and Dalton, 1959) and gills (Vickers: unpublished observations), although the pseudobranch cells of the guppy do not show any vesicle (Copeland reports one in *Fundulus* in his earlier light microscope study, although not in this later study with the electron microscope), and I have never seen any changes in them associated with salt loading. A further point of difference is that the pseudobranch of the goldfish has cells resembling the chloride-secretory type even though the gills seem to be incapable of forming them. How far one should take arguments of this kind is a difficult point; after all the parathyroid gland is derived from branchial arch epithelium, but it would be naïve to argue that it must therefore be involved in sodium and chloride excretion.

The key may lie in the darkening shown by fish which are failing to adapt to salt; this suggests that, even if the pseudobranch is involved in pigmentation changes, salt loading may change its activity, or, at any rate, that the objection may not be an insuperable one.

The distribution of the chloride-secretory cells is potentially wider than hitherto recorded, and this may be significant in several ways: first, it suggests that these cells are ectodermal derivatives; secondly, these cells may be homologous with those in amphibian skin which are responsible for the ability which that tissue shows for the active transport of ions. If this relationship does in fact exist, it would provide useful pointers for further work on both gills and skin. For example, there is evidence that there are two mechanisms present in frog-skin, one responsible for the active transport of chloride and sensitive to adrenalin, and the more conspicuous one transporting sodium and in some way involving choline esterase (Koefoed-Johnsen, Ussing, and Zerahn, 1952; Kirschner, 1953). The so-called chloride-secretory cells apparently contain an esterase which might well be a choline esterase, and Keys found that adrenalin affected chloride excretion in his perfused-gill preparation. Should this be taken to imply that there are two mechanisms in the gills also, and that the chloride-secretory cells are in fact responsible for active transport of sodium? Certainly, Krogh (1939) found evidence for two distinct absorptive mechanisms, one for sodium, the other for chloride, in the gills of freshwater fish. Since the cytological changes of the gill-cells in response to the imposition of ionic work are so clear, it might be possible to decide whether chloride evokes changes in the same cells as sodium, or if only one of them is effective, or if there are two distinct populations of cells, one responding to chloride, the other to sodium. Preliminary experiments to test these points have proved inconclusive because of the difficulty of finding physiologically inert ions to balance the ion to be tested.

A further point arising out of the potentially wide distribution of these cells is that although, if the function ascribed to them is indeed that which they perform, they are extremely active, they would nevertheless appear to be akin to keratinizing, ciliated, or mucous cells, in that they can be formed over a very considerable part of the epithelia of the body, and their functional

differentiation, although highly developed, may thus be a primitive one: the ability to transport ions, especially sodium, must be universal among cells. Further study of the chloride-secretory cell might well throw light on the structural organization underlying ion transport as a general cell phenomenon.

A study is now being made of these cells with the electron microscope, and, until detailed information is available about the structure of the cells and the interrelations of their organelles, there seems to be little value in further speculation as to the special significance of the striking features of the cells, such as the vesicle and the characteristic staining properties.

So far as the original purpose of the study is concerned, these cells appear to be the only ones in the gills whose appearance is compatible with the function of large-scale excretion of salt, and the arguments against such an identification cannot be regarded as outweighing the striking correlation between the ionic load imposed on the gill, and the changes observed in these cells.

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On the Reproduction of *Prionoplus reticularis* (Coleoptera, Cerambycidae), with General Remarks on Reproduction in the Cerambycidae

By JOHN S. EDWARDS

(*From the School of Agriculture, University of Cambridge*)

With one plate (fig. 9)

Summary

Gametogenesis is completed in the pupa in both sexes and the gonads degenerate in the non-feeding adult.

Cyst wall cells, which act as trophocytes, accompany each cyst from an axial germinal region to the radiating follicles. A preliminary account is given of a fibrous structure, the lens of Demokidoff, which overlies the germinal region in the testis lobe.

The morphology of the gut and gonads of the male *Ochrocydus huttoni* are briefly described.

A distinction is drawn between the reproductive patterns of the subfamilies Prioninae and Lamiinae. In the former, where a non-feeding adult is characteristic, gametogenesis is completed in the pupa, after which the gonads degenerate, while in the Lamiinae, where the adult feeds, gamete production continues through adult life. Between these extremes, the Cerambycinae and Aseminae appear to tend toward the prionine condition, and the Lepturinae toward the lamiine.

Introduction

THE biology of the Cerambycidae has a large literature, but little is known of reproduction in the family. The limited information, nevertheless, suggests that *Prionoplus reticularis* White exemplifies a reproductive pattern typical of prionine Cerambycidae. Feeding is restricted to the larval stage; in the short-lived adult the mid-gut is a vestige and the fore-gut a dilated air-sac (Edwards, 1961). Ovulation and spermatogenesis occur early in the pupal stage and the gonads are already regressing when the adult emerges from the log-bound pupal chamber. The newly emerged gravid female is more or less sedentary, assembling males by means of an olfactory attractant; she flies only after part of her heavy load of eggs is laid. Further details of reproductive behaviour are discussed elsewhere (Edwards, 1961).

Material and Methods

Observations were made on live and preserved material collected in the Riverhead State Forest near Auckland, New Zealand, during the summers of 1953–4 and 1954–5. Pupae of known age were obtained from mature larvae which were collected in the field and pupated in the laboratory. Histological material was fixed in aqueous or alcoholic Bouin, sectioned at 8 or 10 μ, and

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stained in Mayer's haemalum and eosin or Mallory's connective-tissue stain. Carnoy's fixative and acetocarmine 'squashes' were used to examine the nuclei of gonad tissue.

Results

Morphology of the reproductive system

Gonadal strands develop rapidly in the prepupal stage; gonad rudiments were not located in dissections of mature larvae. The active gonad reaches maximum development in the pupa and it is mainly on this stage that the following descriptions are based.

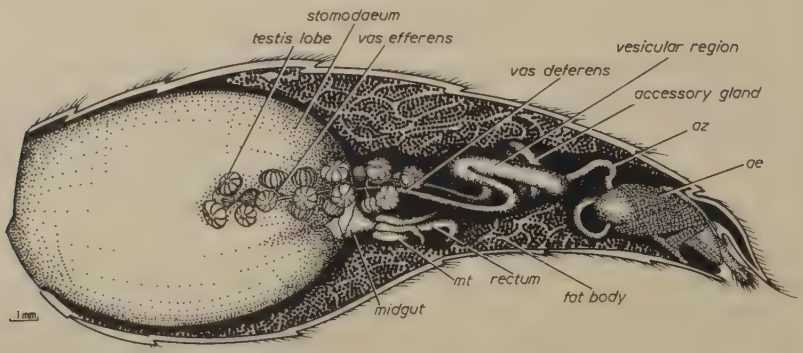

Fig. 1. Gut and reproductive system of a young adult male *Prionoplus*. *ae*, aedeagus; *az*, ductus ejaculatorius; *mt*, Malpighian tubule.

The male (fig. 1). The arborescent testis is composed of 12 to 15 lobes, connected to an axial vas deferens along its anterior third by short vasa efferentia. Each lobe, about 0·75 mm in diameter, comprises 15 follicle sectors arranged about a central cavity in the manner of a mandarin orange. The testis lobes are embedded in massive fat-body that occupies almost the entire pupal haemocoel, and are surrounded by a peritoneum which appears first as a layer 2 or 3 cells thick, distinguishable from the surrounding fat-body by the relative lack of cytoplasmic inclusions.

The peritoneum eventually condenses to form a membranous layer whose originally cellular nature is evident only where small, isolated nuclei remain. The follicular epithelium of the testis which is initially cuboidal also loses its distinctly cellular structure as development proceeds, coming finally to resemble the peritoneum. The development of the spermatic cysts and an apical structure of each testis lobe, comparable with the 'lens of Demokidoff' (Deegener, 1928), are discussed later. The vasa efferentia and terminal vasa deferentia have delicate circular muscle-layers. Posteriorly to the testis lobe region the vas deferens expands to form a storage region. Shortly before uniting with its pair the wall becomes thickly muscular and receives the duct of a crescentic, mesodermal accessory gland. The secretion of the accessory gland is an opaque, finely granular material that passes into the sperm storage region

of the vasa deferentia, where it mixes with the sperm mass. *Prionoplus* does not form a spermatophore.

The muscular ductus ejaculatorius, which reaches a length of 2·5 cm, lies tightly coiled between the apodemal wings of the aedeagus in the resting position. It is continuous with the endophallus, which in the invaginated state lies coiled with the ductus ejaculatorius. In the everted state the endophallus (fig. 2) is seen to consist of 3 regions. The basal part between the median aedeagal valves is more bulbous than the remainder; it terminates where sclerotized processes arise on each side. The lateral plates of these processes re-

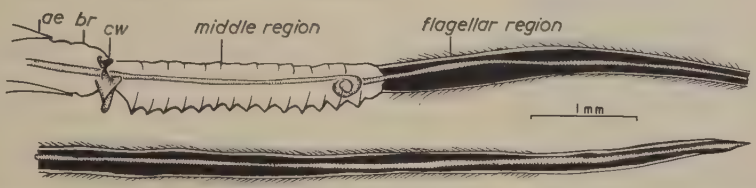

FIG. 2. Everted endophallus of *Prionoplus*. *ae*, aedeagus; *br*, basal region; *cw*, chitinous wings.

semble those figured by Duffy (1953*a*) for *Prionus coriarius*. It has been suggested that these structures act as claspers, but observation of *Prionoplus* when mating did not lend support to this view. It seems more probable that they serve to enclose the invaginated internal sac and retain it within the body by means of retractor muscles passing to the aedeagal wall, the transverse processes forming a 'double door' to the aperture of the aedeagus. The second or middle part of the endophallus is a glabrous tube, corrugated ventrally with 16 backwardly facing transverse ridges, each slightly sclerotized at the apex. This region is the first to be evaginated during pairing and serves to hold the endophallus within the ovipositor during eversion of the flagellum. Fine retrorse hairs that cover the flagellum except at the tip evidently serve to prevent it from moving back through the vagina during the movements of ejaculation. It is retracted after coitus by reinvagination. Muscle-strands from more distal points insert on the mid-ventral base of the aedeagus, and from proximal points on the anterior apodeme of the phallobase and the aedeagal wings.

Abdominal contractions that appear to be associated with the development of a hydrostatic pressure in the haemocoel for evaginating the flagellum are seen during the early stages of pairing.

The genitalia and genital musculature will be dealt with in a further paper.

The female (fig. 3). The ovaries of the adult female are more or less obliterated by the egg masses within the distended lateral oviducts. The following description therefore refers mainly to the pupal organs.

The paired ovaries, each composed of 42 to 45 polytrophic ovarioles, lie dorsolaterally in abdominal segments back to the 7th, where they attach ventrolaterally to the 7th sternite. The terminal ovarian filaments on each side are united to form paired ovarian ligaments attached to the metathoracic phragmata. During the approximately 10-day prepupal stage the external

genitalia are formed by invagination and the bifid genital duct so formed unites anteriorly on each side with the calyces, forming the cuticle-lined lateral oviducts of the mature female. The primary connexion of the mesodermal gonoduct with sternum 7 is retained throughout life as a bundle of degenerate muscle-tissue and tonofibrillae which provide a posterior anchorage to the distended calyces.

FIG. 3. Reproductive system of a female pupal *Prionoplus*. *lo*, lateral oviduct; *spv*, spiculum ventrale.

In the early pupa the calyces are stellate in cross-section, expanding as they receive eggs, and displacing fat-body until they meet in the mid-line. Finally, towards the end of the pupal period, they come to occupy the greater part of the abdominal cavity. The wall is membranous, the basement membrane and peritoneum forming the greater part of its thickness. The lateral oviducts which open to the vertical mesial walls of the calyces in segment 6 are distensible muscular ducts in which the intima is clothed with cteniform, scale-like projections (fig. 4). The spines of each scale are hollow at the base but do not appear to open at the tip. Since the membranous calyx can play no part in the extrusion of eggs to the lateral oviduct, this must be effected by the muscular oviduct walls, the spinose intima serving to grip the egg. The median oviduct is notable for the form of the muscularis, in which a strong circular layer fuses with the intima along two diametric lines (fig. 5). Between the dorsal and ventral transverse crescentic muscle-bands and the epithelium lies a group of longitudinal muscles which have no connexions with the circular muscle and which insert to the intima at scattered points. The vagina within the ovipositor has a simple circular muscularis and resembles its male homologue, the ductus ejaculatorius. The accessory structures of the female reproductive system are associated with the anterior end of the median oviduct (fig. 6) where it fuses with the lateral oviducts in a muscular region which also receives extrinsic retractor muscles from the spiculum ventrale, sternum 8, and apodemal surfaces of segment 9 on the inner wall of the ovipositor.

The spermatheca and bursa copulatrix arise together in a muscular stalk, the bursa soon separating to form a finger-like lobe. The apex of the spermatheca is reflected and attached to the spermathecal stalk by muscle-fibres (fig. 7). In this region the outer wall is rather more heavily cuticular than the inner wall of the curve. Manipulation of fresh material suggests that contraction of these muscles, for which the curved cuticular wall acts as antagonist,

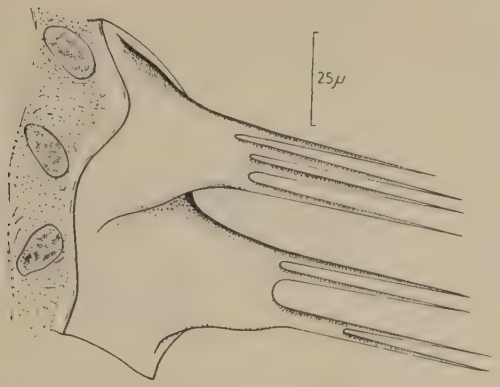

FIG. 4. Spines of lateral oviduct intima.

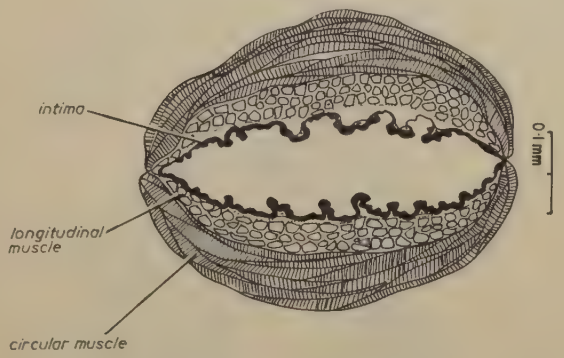

FIG. 5. Transverse section of the median oviduct in the region of divided circular muscularis.

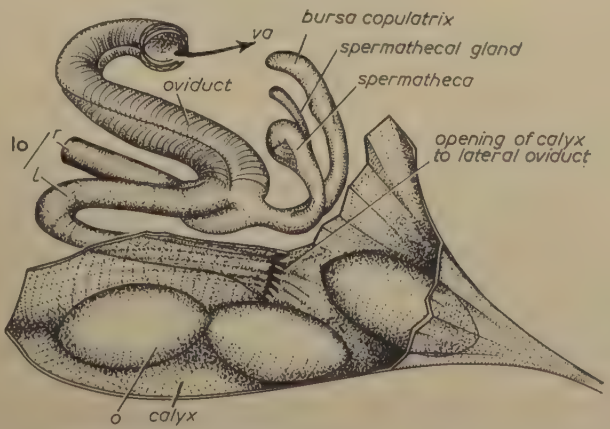

FIG. 6. Oviducts and associated structures in an adult female *Prionoplus* *lo*, lateral oviducts (*l*, left; *r*, right); *o*, ovum; *va*, vagina.

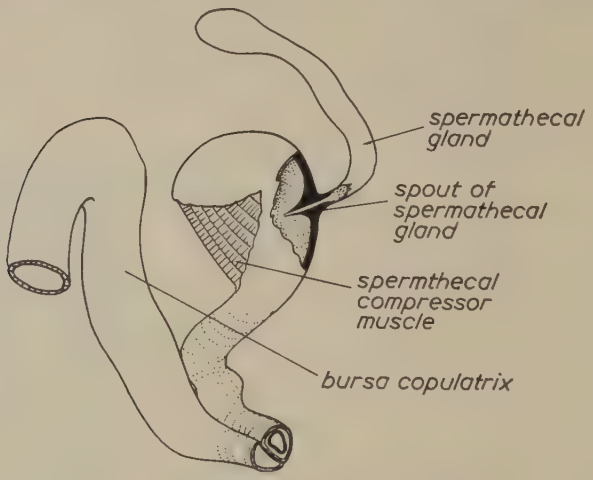

FIG. 7. Spermatheca and bursa copulatrix.

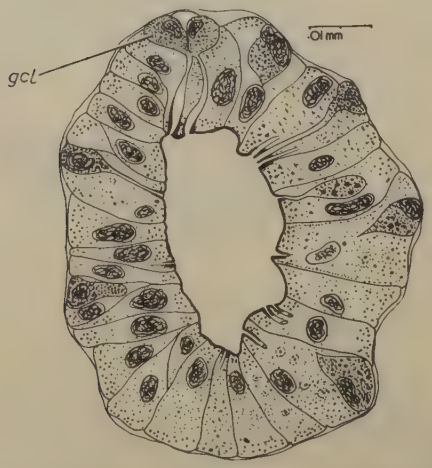

FIG. 8. Transverse section of spermathecal gland. *glc*, gland cell.

serves to express sperm from the spermatheca. The acutely tapered spout of the spermathecal gland evidently prevents entry of sperm during the compression stroke, and possibly releases its secretion during the recovery stroke. The precise function of the compressible spermatheca in *Prionoplus* remains problematical, since the entire bursa copulatrix, spermathecal duct, and common duct are usually packed with spermatozoa in mature females.

Ductules of cellular glands are common in the wall of the spermathecal gland (fig. 8).

The long ovipositor bears a pair of subapical clavate lobes, each of which bears a terminal circlet of sensory setae surrounding a group of longer sensory hairs that are employed during selection of the oviposition site.

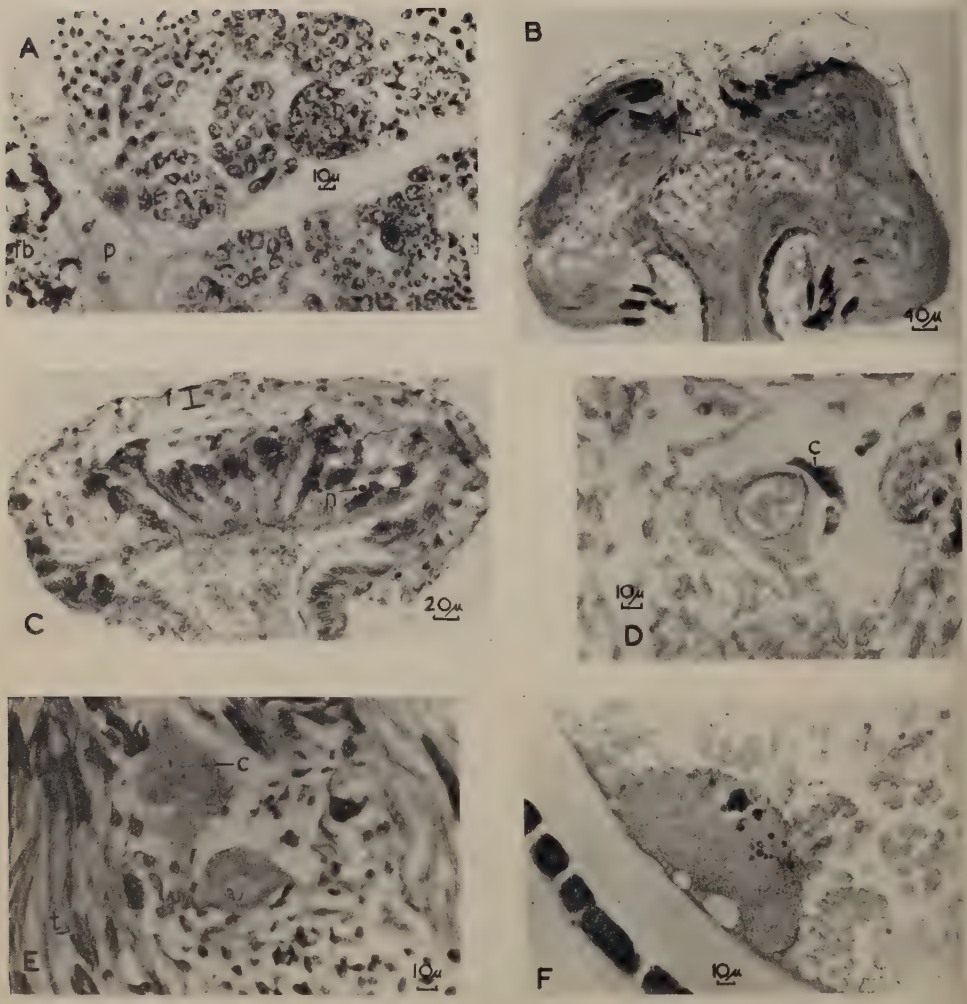

FIG. 9

J. S. EDWARDS

Development and senescence of the testes

The testes of *Prionoplus* are active only for a short time in the life of the individual. After rapid growth in the prepupa they reach maximum activity early in the pupal stage, and maturation of all the spermatozoa is complete before emergence of the adult.

Development of spermatozoa. The germarial region is subapical and extrafollicular, lying under the 'apical lens' to be discussed below. Cyst and germ cells move into the radiating follicles where growth and maturation takes place (fig. 9, A). Cyst formation is most rapid in the late prepupa, giving rise to a mass of cysts which are almost synchronous in development, though for some reason not yet understood the more axial and more peripheral cysts mature at a slower rate than those lying between them, and small groups of adjacent cysts may develop out of phase with those surrounding them. The majority of cysts contain primary and secondary spermatocytes by the 5th to 7th pupal day and spermatids are present by the 7th to 10th day. No dividing cells were found in acetocarmine 'squashes' of testis tissue older than 10 days. 128 spermatids are formed in each cyst. Discharge of spermatozoa to the axial cavity of the testis lobe, and so to the vas efferens, usually takes place in the late pupal or preimaginal stage (fig. 9, B). Thereafter the testis lobe decreases in diameter from a maximum of 0·75 mm to 0·4 mm or less (fig. 9, C).

By the time the hardening and darkening of the adult cuticle has occurred much of the sperm mass has reached the now dilated lower region of the vasa deferentia, and from this stage on the testis wall undergoes degenerative changes.

Up to the time of their release from the cyst the spermatozoa are linear, but as they break from the cyst and arrive in the lumen of the lobe the tail develops an open spiral form with a periodicity of about 10 μ.

Supporting tissue. The nuclei of the two cyst cells or trophocytes of each spermatic cyst are prominent and densely basiphil from the time of their arrival in the follicle. When the cyst elongates during spermatid formation, the trophocyte nucleus also lengthens and becomes flattened closely over the spermatid bundle (fig. 9, E). Unlike the cyst cells of the rutelid beetle *Popillia*

FIG. 9 (plate). Photomicrographs of material fixed in Bouin; paraffin sections, 10 μ.

A, transverse section of an early pupal testis of *Prionoplus*. Mayer's haemalum, eosin. *fb*, fat-body; *p*, peritoneum.

B, median longitudinal section of newly emerged adult *Ochrocydus huttoni*. Mayer's haemalum, eosin. *l*, lens.

C, median longitudinal section of testis of adult *Prionoplus* in advanced senescence, showing apical cap composed of lens and overlying connective tissue. Below the cap is a syncytial layer containing endomitotic nuclei. Mayer's haemalum, eosin. *f*, open connective tissue; *t*, connective tissue containing tracheae; *n*, degenerating trophocyte nucleus.

D, median longitudinal section of a late pupal testis of *Prionoplus*. Mallory's triple stain. *c*, connective tissue.

E, median longitudinal section of the lens in a late pupal testis of *Prionoplus*. Mayer's haemalum, eosin. *t*, elongated, flattened trophocyte nucleus.

F, longitudinal section of the germinal vesicle of a *Prionoplus* oocyte, showing nucleolar buds. The follicular epithelium has separated from the oocyte. Mayer's haemalum, eosin.

japonica described by Anderson (1950), which are said to accompany the spermatozoa from the testis lobes, continuing their nutritive function, those of *Prionoplus* do not leave the lobe. Their exact fate has not been determined but it is thought that the majority break down when the bundles of spermatozoa leave the cyst, though a few may remain in a group of large endomitotic nuclei in the coenocytic tissue of the senescent testis that lies under and in contact with the apical lens.

The apical lens. An apical cell of a Versonian type (Snodgrass, 1935) is not present in the testis of *Prionoplus* or *Ochrocydus*. The apical region is, however, occupied by a lenticular body similar to that briefly described by Demokidoff (1902) in the testis of *Tenebrio molitor* and termed the 'lens of Demokidoff' by Deegener (1928). The composition and function of the lens requires further examination, but a preliminary description is appropriate here.

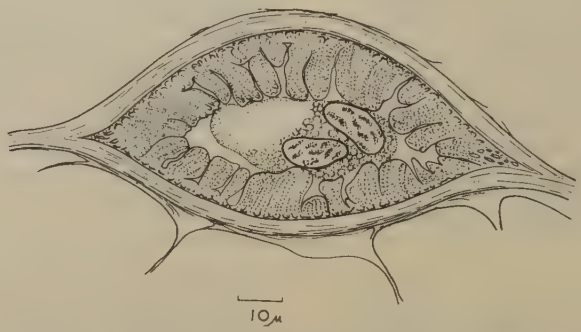

FIG. 10. The lens of a late pupal testis of *Prionoplus*.

In the active testis of the early pupa the lens overlies the germarial region. It is evidently derived from the basement membrane of the testicular epithelium, which is thickened to form a material of fibrous appearance staining weakly with eosin and giving a strong orange-red with Mallory's triple stain. Within a fibrous coat a much-folded layer, with a radiating fibrous appearance and similar staining properties to the laminate coat, surrounds a central cavity containing several nuclei. The nuclei are not markedly endomitotic. Above the lens, but not in contact with it, is a loose mass of connective tissue.

In the late pupa the lens (figs. 9, D, E; 10) is more convex, and the central cavity larger. In *Prionoplus* it is about 40 μ deep and 50 to 60 μ in greatest diameter. The lacuna, 25 μ in diameter, contains about 10 nuclei.

In the adult testis the lens material extends over the surface of the lobe as a cap up to 150 μ in diameter. It is here composed of two layers, both giving a strong orange-red colour with Mallory's triple stain. The outer region, 20 to 30 μ thick, apparently derived from previously blue-staining connective tissue originally overlying the testis, contains tracheoles and scattered nuclei. The more homogeneous inner layer, the lens proper, is seldom more than 10 μ thick. A ring of open connective-tissue, packed with convoluted tracheoles,

surrounds the lens. Below, separating it from the sperm-filled lumen of the shrunken lobe, is a layer of syncytial tissue containing large endomitotic nuclei, degenerating nuclei in which the chromatin has clumped, and numerous trapped spermatozoa. Tracheoles traverse the tissue, and there appear to be ductules passing from the endomitotic nuclei to the lumen of the lobe. The tissue appears to be undergoing autolysis; it is entirely resorbed in aged males.

The lens bears little resemblance to a typical apical cell, and its relationship to the germinal tissue in early stages of the testis and during resorbtion in the adult is not clear. Demokidoff's view that in *Tenebrio* it is simply a structural support (*Stützorgan*) from which connective tissue carrying tracheoles enters the testis before pupation, probably only partly accounts for its function.

Development and senescence of the ovaries

Like the testes, the ovaries are only active for a short time. The pattern of their development and regression parallels that of the testis. Delicate ovarian filaments arise in the prepupa, and by the 5th pupal day tubular germaria are present in the 2nd and 3rd abdominal segments. At 5 to 7 days several ova have enlarged in most ovarioles, producing about 400 mature eggs. More apical ova and oocytes are compressed against the metathoracic phragmata as the calyces fill and press the germarial region forward. These do not develop when oviposition begins, but are resorbed, so that there is virtually no remnant of the ovarioles in the mature adult.

In the active ovariole of the early pupa, oocytes are differentiated toward the lower end of the germarium and do not appear to be accompanied by trophocyte cells when they leave the germarium. Four to 5 oocytes in the neck, above the first separate follicle, increase from about 16 μ to 130 μ in diameter. Their germinal vesicles are central.

The follicular epithelium surrounding the enlarging ovum is composed of columnar cells with strongly basiphil cytoplasm and large endomitotic nuclei. Simultaneously with the beginning of yolk deposition the germinal vesicle moves to the wall of the primary oocyte and nucleolar budding gives rise to a number of vesicles that lie toward the inner margins of the germinal vesicle. Yolk granules have a radiating orientation about the corrugated inner surface of the germinal vesicle, suggestive of its role in yolk synthesis (Bonhag, 1958). (See fig. 9, F.)

The ovariole epithelium between follicles is columnar and extends to the centre of the ovariole but in these regions it is markedly less basiphil than the cubical epithelium surrounding the developing ovum and does not appear to have a nutritive function.

A note on Ochrocydus huttoni

The morphology of the adult gut and testis of the cerambycine *O. huttoni* closely resembles that of *Prionoplus*. The mid-gut is a functionless vestige and

the fore-gut dilated and membranous. As in *Prionoplus* the cryptonephric Malpighian tubules and rectum are little changed from the pupal condition apart from loss of muscle. The testis on each side comprises 22 to 24 lobes, arranged as in *Prionoplus*, each with 20 follicles. Maturation of spermatozoa is complete at emergence of the adult (fig. 9, B).

Cerambycid reproductive cycles

Linsley (1959), in his recent review of cerambycid biology, states that 'although some Cerambycidae (e.g. *Hylotrupes*, *Stromatium*) require little or no food in the adult stage, for most species some feeding appears to be an essential prerequisite to egg maturation and oviposition'. It is generally agreed, however (e.g. Butovitsch, 1939; Craighead, 1923; Duffy, 1953*a*, *b*), that the majority of Prioninae and a large number of Cerambycinae require no food as adults. The food of adult Lamiinae does suggest that they require a nitrogen source as adults for reproduction.

It is to be expected that this contrast between a long-lived feeding adult and a short-lived non-feeder should be reflected in the reproductive pattern, though this does not appear to have been recognized previously. It has been shown above that in two species, one a prionine and the other a cerambycine, gametogenesis is compressed into a comparatively short period of intense activity in the pupa and that the gonads of the adult are senescent. Silantjew (1908), found the reproductive organs of the cerambycine *Stromatium fulvum* 'fully developed' at emergence and observed that the digestive organs were weakly developed and never contained food. Ritchie (1920), on the contrary, noted that the reproductive organs of the lamiine *Saperda cacharia* (an adult feeder) are 'not quite mature' on issue of the imagines but ripen in a short time.

Ehara (1951), who examined the histology of the adult testes of 41 species of Cerambycidae from a taxonomic point of view, distinguished the Cerambycinae and Lepturinae in which he found only spermatozoa and spermatids, from the Lamiinae in which meiotic divisions were to be found throughout the adult life.

On the basis of these observations its seems possible to make a clear distinction between typical prionine and lamiine Cerambycidae. In the former, gamete formation is completed in the pupal stage in both male and female, and the adult does not feed. In the Lamiinae, on the contrary, the adult continues to feed and gamete formation continues throughout the longer adult life. Other subfamilies lie between these extremes. There is probably considerable variation within each group but the available data suggest that the Cerambycinae and Aseminae tend toward the prionine pattern and the Lepturinae toward the lamiine pattern.

I thank Dr. J. G. Pendergrast of the Department of Zoology, University of Auckland, for helpful discussion during this study.

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Properties of the Wall of *Leucosolenia variabilis*

I. The Skeletal Layer

By W. CLIFFORD JONES

(*From the Department of Zoology, University College of North Wales, Bangor*)

With three plates (figs. 2 to 4)

SUMMARY

Pieces of the wall were photographed while being stretched by a pair of light weights. The extension took place slowly and removal of the weights was not followed by appreciable elastic recoil. On the photographic records, however, slow recoil could be detected in places. The slowness of both extension and recoil are attributed to the mesogloeal matrix and its embedded spicules; the changes in shape necessitated plastic deformation of the matrix.

Changes in the arrangement of the spicules on the photographic records confirm that the matrix underwent plastic deformation, while firmly embedding the spicules. Also the matrix yielded in places, whereupon the epithelia in the vicinity became greatly stretched and recoil occurred on each side of the region concerned.

In some parts of the oscular edge the epithelia contracted and caused the spicules to become crowded together.

Small developing spicules often underwent rotation on the stretching pieces and in some cases the rotation exceeded 180°. While their founder calcoblasts were probably attached to the epithelium which joins the gastral ends of the pores together, the attachment cannot be regarded as permanent, since the rotation, and also spicule growth, require that the calcoblasts at the tips of the rays should be able to move over the surface of the epithelium. The rotation of some developing spicules was associated with a displacement relative to the mesogloeal matrix.

The photographed pieces could not be held for a sufficient time (over 20 h) for new patterns of orientation to be shown by the developing spicules, but on one unphotographed piece this was possible, and small spicules oriented in the opposite direction to the fully formed ones were found near the basal edge, just behind a region of spicule crowding at the edge itself.

The relevance of these observations to the mechanism of spicule orientation is briefly considered.

INTRODUCTION

IN 1959 I described a method whereby stretched pieces of the wall of *Leucosolenia variabilis* could be photographed alive at intervals (Jones, 1959). The photographic records thereby obtained were used to determine the rates of growth of the spicule rays, but they also provided information about other features of the wall, which will be presented in this and in a subsequent paper. The information largely concerns the skeletal layer (the mesogloea and its embedded spicules) and the tissue lining the cavity of the oscular tubes (the choanocytes and the porocytes), and is of particular interest in connexion with the mechanism of spicule orientation. To explain this I have suggested that during the growth of the oscular tube, there is an orienting movement of the mesogloeal matrix past the spicule-forming calcoblast sextets, which lie in contact with the internal epithelium (Jones, 1954*a*, 1958). For this hypothesis

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to be verified it is necessary to prove that the mesogloeal substance has plastic properties and, at the same time, sufficient viscosity to maintain the oriented sextets in their orientation while the spicules are being formed. It is also necessary to demonstrate that the epithelium to which the sextets are attached has the required growth properties to bring about the shear of the mesogloeal matrix; and furthermore the necessary stability to anchor the calcoblasts while they are experiencing the shearing movement of the matrix.

Method

Details of the method have already been published (Jones, 1959). An oscular tube of *L. variabilis* (Haeckel) was excised from the sponge and cut lengthwise so that the cylinder could be unrolled and then mounted with each cut edge between the jaws of a glass clip. The clips were placed in a dish of sea-water under a microscope and steadily drawn apart by means of small weights (up to 0·7 g on each side). At intervals the pieces were photographed with a 2/3-in. objective and a Leitz 'Makam' camera.

Results

Changes in shape of the pieces

The continuous application of tension across the pieces caused them to widen slowly and, at the same time, to shorten longitudinally, the front and back edges arching inwards from clip to clip. The effect was somewhat like that produced when a broad elastic band is stretched, but the edges did not arch inwards uniformly; here and there projections supported by the propping action of interlocked groups of spicules were present or appeared. The pieces also differed from a stretched elastic band in that the extension under constant load took place very slowly (one piece had only doubled its width after 1½h), and also there was no appreciable elastic recoil when the stretched pieces were removed from the clips. I have already stated (Jones, 1956) that the pieces cannot be extended by the sudden application of tension, and that excessive tugging by means of a pair of needles merely ruptures the wall. Thus the slow extension of the mounted pieces must have involved structural alterations of some kind, and this is confirmed by the absence of appreciable recoil when the pieces were released after stretching. Despite the considerable extension achieved (after 17 h one piece had become as much as 9 times its original width), removal from the clips was always followed by slow changes, the pieces undergoing the shrinking and curling behaviour already described for unstretched parts of the wall (Jones, 1957). Slow local shrinkages were also visible on the photographic records of the mounted pieces. In fig. 1 are shown the outlines of one of these pieces after successive intervals of time. The positions of marker spicules are also indicated, and a comparison of B, C, and D in fig. 1 reveals that a portion of the anterior edge for a time became more extensible than the rest, and permitted recoil to occur in the adjacent region. The recoil, or shrinkage, continued for several hours. Greater extensibility was also usually found in the parts of the wall which had been initially squashed

between the jaws of the clips. Thus considerable areas were drawn from between the jaws as stretching proceeded, and in time such areas tended to rupture. The undamaged central regions, on the contrary, did not extend so much. The extension was accompanied by an increase in the surface area, as is shown by the increase in area of the polygon drawn through the marker spicules in fig. 1, A–D. This indicates that the wall was becoming thinner, as did also the improvement in the ability to focus simultaneously the spicules lying at different levels in the mesogloea.

The slowness of the stretching and shrinking processes is probably due to the presence of the spicules and the supporting properties of the mesogloeal matrix. The latter is normally a stiff gel which firmly embeds the spicules. It can be softened and swollen by treatment with potassium nitrate solution, but immediately stiffens again on being returned to sea-water (Jones, 1956). Since the spicules embedded in it are rigid structures, the changes in shape of the pieces during stretching or shrinking must entail plastic deformation of the matrix, a slow process for a gel. Changes in the arrangement of the spicules will now be considered in more detail.

Changes in the arrangement of the tri- and quadri-radiate spicules

The spicule arrangement broadly speaking followed the change in outline of the piece as this was stretched (fig. 1). Thus the spicules tended to separate in the direction of the applied tension, and tended to approach one another in the direction at right angles to this. The movements, however, were by no means uniform, some of the spicules showing larger, and others smaller, displacement or rotation in relation to their neighbours, or even approaching one another along the direction of applied tension. Some examples will be seen shortly. This lack of uniformity was only to be expected, since the forces acting on the spicules were so very variable, because of the shape of the spicules and differences in their position and arrangement. The rays of the tri- and quadri-radiate spicules do not all lie in the same plane, even when the gastral rays of the quadriradiates are excluded. The paired oscular rays are slightly inclined so as to fit the curvature of the wall of the oscular tube (Jones, 1954*b*). Thus when the wall is stretched out flat, one or both of these rays must be directed towards the choanoderm, and may come into contact with it. Other parts of the spicule may be in contact with the pinacoderm, or with the rays of adjacent spicules, while calcoblasts may or may not ensheath the tips of the rays and extend cytoplasmic processes to adjacent structures. Thus the forces acting upon the spicules as the wall is stretched will vary from one spicule to the next, and even if the contacts were the same for each spicule, there would be local differences in extensibility in the two epithelia, and heterogeneities in the mesogloeal matrix, to prevent uniform changes in the spicule arrangement. When only those spicules were considered which lay in contact with the internal epithelium along all 3 rays (that is, developing or recently formed spicules), it was usually found that they formed a constellation in which the positions of the central junctions of the spicules tended to alter uniformly as

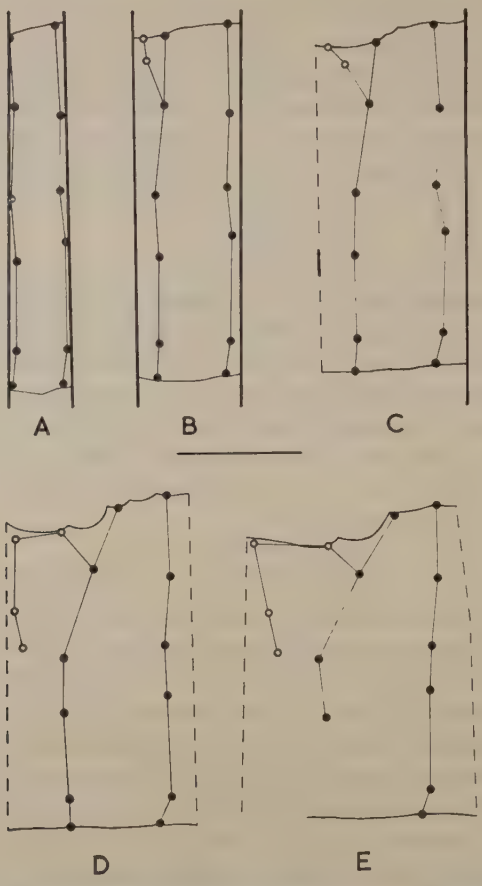

FIG. 1. The changes in outline produced by stretching a piece of the wall. The diagrams were drawn from measurements made on photographs taken at 0, 2, 5, 8½, and 11 h respectively after the piece had been mounted in the stretching apparatus. The oscular rim is at the top end of each diagram. The thick lines represent the edges of the clips used to hold the piece, and the broken lines indicate the outer limits of the photographed area. The positions of marker spicules are shown by the points, those with clear centres representing spicules which have been drawn from beneath the clips. The points have been interconnected by thin lines to facilitate the identification of the spicules. In the centre the short line indicates a length of 1 mm and shows the direction of the applied tension. Note that the transverse stretching is accompanied by longitudinal shrinkage, and that the left part of the oscular rim was more extensible than the right (B–C), which underwent contraction or recoil (B–E).

stretching proceeded. Even here, however, irregularities occurred, as would be expected, since the spicules are rigid structures and this would necessitate readjustments of contact as the epithelium altered its spatial dimensions with the stretching process. Three particular areas will now be considered in detail.

The region depicted in fig. 2

As stated above, part of the anterior edge of the piece concerned in fig. 1 became greatly stretched. Successive photographs of this part are shown in

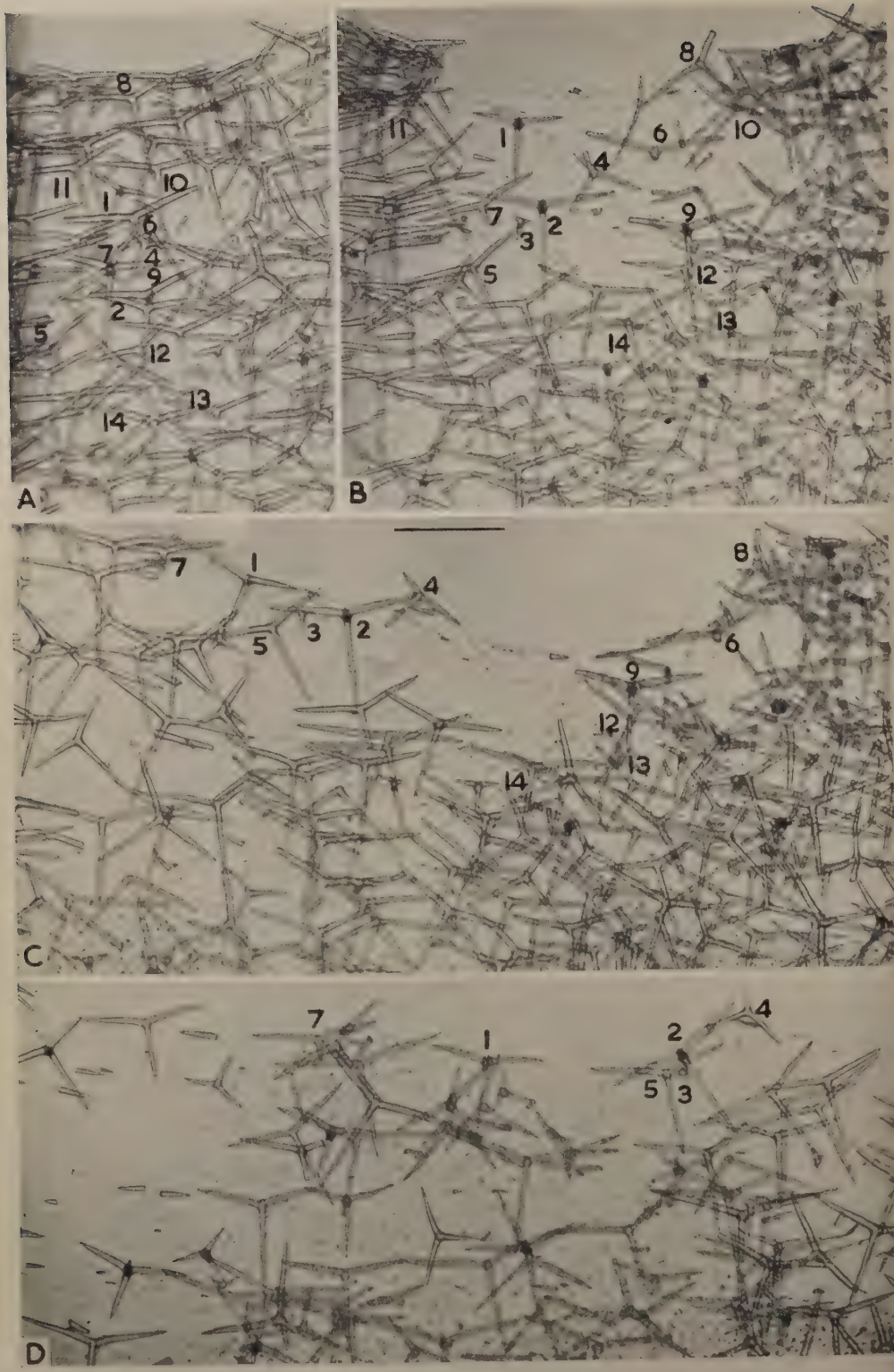

FIG. 2

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fig. 2, and by comparing fig. 2, A and B, one can see that most of the spicules moved off to the right and left, leaving an area relatively free. In this area the two surface epithelia were intact, as can be seen on the subsequently fixed and stained preparation. Of the spicules left in the area, two were quadriradiates (spicules 1 and 2), of which one was still in course of growth (sp. 1). One would expect these quadriradiates to be anchored to the upper (internal) epithelium by their gastral rays at least. About half-way down the basal (unpaired) ray of spicule 2 can be seen a cluster of cells, probably calcoblasts, which would also be in contact with the upper epithelium. This cluster does not move far from spicule 2, and the same is true for spicules 3, 4, and 5. The other spicules on each side have travelled farther. Thus 6 has moved more than 4, and 7 has passed 3 on the left; while 8, which is clearly anchored to the epithelium by a projecting ray, and 9, another quadriradiate, have not travelled as far as, for example, 10. These examples suggest that whereas some of the spicules are anchored to the upper epithelium, there are many which are not. Of the latter some also do not appear to be anchored to the lower epithelium (pinacoderm) either. Thus the left oscular ray of 6 and the right oscular ray of 7 begin on the sides opposite to those towards which they move, and these rays clearly must have passed very close to one another. There is no possibility of anchorage to an epithelium in their case, and since the spicules to which these rays belonged separated with little change in their orientation, it would appear that the mesogloeal matrix had yielded, or ruptured, along a line a little to the right of that joining spicules 1 and 2, producing two separating parts, one embedding most of spicule 7 and the other most of spicule 6. These two parts separated not only as a direct result of the applied tension, but also as a result of recoil or contraction in the stretched tissue. The latter is indicated by the crowding together of the spicules on each side of the extended region. This spicule aggregation continued slowly for several hours (fig. 1).

The spicules did not betray any violent changes in orientation in the recoiling areas, but some rotation was shown by spicules near the free edge, for example, spicule 10 on the right and 11 on the left. Those on the left rotated anticlockwise and those on the right clockwise, except 8, which was anchored to the epithelium and was presumably turned in the opposite direction by the movement of the mesogloeal matrix against the basal ray; the matrix presumably recoiled farther than the epithelium in the region of spicule 8, because the epithelium still remained continuous, though severely stretched (as shown by the inward arching of the anterior edge) across the region of weakness. The spicule rotations suggest that the yielding or rupture of the mesogloeal matrix

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FIG. 2 (plate). A–D, part of the oscular rim of the piece concerned in fig. 1, photographed respectively at 2, 5, 8¼, and 11 h after mounting. The numbers denote spicules mentioned in the text. It can be deduced from the changes in the spicule pattern that the mesogloea ruptured, or yielded, along a line just to the right of that joining spicules 1 and 2, and that the tissue then recoiled on each side, leaving the severely stretched epithelia and some attached spicules in between (A–B). Continued stretching (B–D) resulted in spicules being brought to the edge from positions previously behind. Further details in the text. The line at the top of C represents 100 μ.

started at the edge (where the strain would be greatest (Treloar, 1949, p. 83)) and then proceeded inwards.

As stated above, the transverse stretching of the piece was accompanied by a longitudinal shortening. The latter is evident when similar parts of A and B in fig. 2 are compared; the spicules tended to approach one another in the longitudinal direction. This is evident also in the regions of recoil, possibly because the yielding occurred late in the time interval between the two photographs. Longitudinal extension in the recoiling areas would also be hindered by the tension in the epithelium at the edge as stretching continued.

Relative displacements in the longitudinal direction occurred with some spicules, for example spicule 12 and the growing spicule 13. Both moved nearer to the anterior edge than the adjacent spicules, spicule 13 more than 12. Spicule 14 is also of interest; the tip of its right oscular ray remained in contact with a particular region of the basal ray of spicule 12 while it moved a long way to the right. This seems good evidence that spicule rays may at times be joined together by inextensible bonds, possibly submicroscopic collagen fibres, since hydroxyproline is a constituent of the mesogloeal matrix (Evans, 1958). Such instances are rare and the connexion between 12 and 14 seems to have been broken by the time the photograph shown in fig. 2, C was taken.

In fig. 2, C it can be seen that spicule 13 has rotated towards the area of shrinkage on the right, like other spicules in the vicinity, but the quadriradiate 9 has not rotated, presumably because its rays are attached to the upper epithelium. It can also be seen that the quadriradiates 1, 2, and 9 have been brought close to the free edge by the continued stretching. This process must have been accompanied by a rearrangement of the cells in the epithelia near the edge, since epithelial cells must likewise have been brought to the edge from the flat surface behind. Spicules 3, 4, and 5, and the calcoblast cluster, still retain their association with 2, while a considerable amount of stretching has occurred on the left, 7 having reached the edge beyond 1. On the right the crowded mass of spicules provided the rigidity necessary to prop out the edge a considerable distance.

Fig. 2, D shows a later condition. This print has been inclined relatively to 2, C to enable it to be fitted in. The picture is again markedly different. Spicule 5 now seems to have lost its relationship with 2, and as the latter moved to the left it has carried the epithelium over the ray of 5, which projects in fig. 2, C. Spicule 5 was now presumably free in the mesogloea and its displacement relative to the epithelium has rotated the small spicule 3. Farther left, 7 has continued to overtake the adjoining spicules. Its right oscular ray pierces the epithelium in fig. 2, C, so that it now was attached to the epithelium at the edge. Spicule 1 was likewise carried to the left by the extension of the epithelium.

The varied spreading of the spicules, some crossing over or under others, reflects differences in anchorage or drag, some spicules being influenced more by the epithelia and others more by the mesogloeal matrix. As these layers extended they would move relatively to one another in places, owing to varia-

tions in extensibility. The rotation and relative displacements of the rigid spicules confirm that the mesogloeal substance must be capable of plastic deformation.

The region depicted in fig. 3

A recoiling region will now be considered. Fig. 3, A, B, shows such a region, photographed at $9\frac{3}{4}$ and $15\frac{3}{4}$ h respectively after the initial application of tension. It can be seen that the spicules became very closely tangled together, far more in fact than at the onset of stretching. The wall was very thin in the stretched regions, but in the region of contraction the aggregation of spicules increased the thickness considerably. As many as 6 spicule rays overlay one another in places. Many rays lifted up the epithelia, and some projected through, enabling the epithelia to draw the spicules together.

The aggregation of the spicules was here caused by a contraction of the epithelia. This is proved by the movement of spicules and fragments that were anchored to the upper or lower epithelium. The relationships between the spicules and the epithelia for the photographs in fig. 3 can be determined with reasonable accuracy, for the piece was fixed and stained with picrocarmine only $1\frac{1}{2}$ h after that of fig. 3, B. Thus the broken-off rays 1, on the left, and 2, on the right, were attached by their broken ends to the upper epithelium. In both cases these ends were not only in contact with the epithelium, but there were epithelial cells associated with them. The tapering ends of these rays, on the contrary, projected downwards through the mesogloea. Both rays rotated considerably in the time between A and B of fig. 3, ray 1 clockwise and ray 2 anticlockwise. Their rotation thus shows that the upper epithelium moved towards the area of contraction to a greater extent than the underlying mesogloeal matrix.

Fragment 3 confirms this. In the fixed preparation it is in contact with the upper epithelium over practically its whole length. As can be seen in the photographs (fig. 3, A. B), it moved farther than the neighbouring small spicule, which seems to be attached by only the right oscular ray to the upper epithelium. Presumably the tapering shape and favourable orientation of the fragment facilitated its greater movement by ensuring a minimum of resistance. Broken rays can certainly travel far through the mesogloea when appropriately oriented. For example, fragment 7 was in contact with the upper epithelium along its whole length and became greatly displaced to the right with respect to the spicules in its vicinity. The triradiate 8, situated at the same level and also in contact with the upper epithelium, also moved in the same direction relatively to the adjacent spicules.

The pinacoderm (lower epithelium) was also contracting in the region of shrinkage. The monaxon 6 is attached to this layer by a number of cells in the permanent preparation, and it moved more or less like the adjacent spicule 5, which has its rays in contact with the upper epithelium over their whole length. Spicule 4 also lies against the pinacoderm, except its left oscular ray, which projects up through the mesogloea. These spicules (4, 5, and 6) have

been drawn towards the anterior edge by the longitudinal shortening of the piece, and so brought into the shrinking area.

These examples prove that the epithelia were contracting in the area of spicule aggregation, either by developing tension themselves, or as a result of elastic recoil from a stretched condition. There is no evidence for a contraction of the mesogloeal matrix here, though the movement of the spicules would no doubt tend to push the matrix along as well. It is also clear that spicules can be attached to the epithelia and can be displaced relatively to the mesogloeal matrix.

The collar-cells were but little affected by the stretching process; they remained roughly circular in outline and roughly similar in size on the stretching pieces, as can be seen by comparing A and B in fig. 3. This indicates that they can adjust their position on their substratum, and also their contacts one with another, as the piece is stretched. In fig. 3, A and fig. 4 large gaps can be seen in the choanoderm, and these tended to disappear by the movement of choanocytes across them. I have already given evidence in support of the view that the porocytes are interconnected beneath the bases of the collar-cells (Jones, 1957), and it would appear that the collar-cells are able to move about on the epithelium so formed. Further evidence will be given in a subsequent paper.

The rotation of small spicules

Some small spicules rotated through a considerable angle as the piece was stretched, as can be seen in fig. 4. Another example, in which the rotation exceeded 180°, will be shown in a subsequent paper. All these spicules were growing during the experiment, and since developing spicules lie immediately beneath the choanoderm (Minchin, 1908; Jones, 1954*b*), it can be assumed that their founder calcoblasts were in contact with the porocyte epithelium which exists, I believe, just beneath the choanocytes. From the ability of the developing spicules to rotate, and from the necessity for the calcoblasts at the tip of the growing rays to move, it is clear that the contact can involve no more than a temporary attachment. Nevertheless, contact between the epithelium and the founder calcoblasts seems essential for normal development. Growing spicules were invariably found to be in contact with the upper epithelium on the subsequently fixed pieces, while many of the small, non-growing spicules had lost this contact completely. In some cases a cessation of growth was associated with the intrusion of a spicule ray between the developing spicule and the upper epithelium. On the other hand, some spicules ceased growth prematurely, even though they retained some contact with the upper epi-

FIG. 3 (plate). A, B, the right side of the oscular end of a considerably stretched piece, photographed at $9\frac{3}{4}$ and $15\frac{3}{4}$ h respectively after stretching began. As can be seen, the spicules here became crowded together, and it can be deduced that a contraction of the epithelia was responsible (see text). The numbers indicate spicules that are mentioned in the text. Note the retreat of the choanocyte boundary relative to the spicule pattern. At the bottom right-hand corner is a gap caused by the middle part of the piece slipping free from the clip used to hold it. The line represents 100 μ.

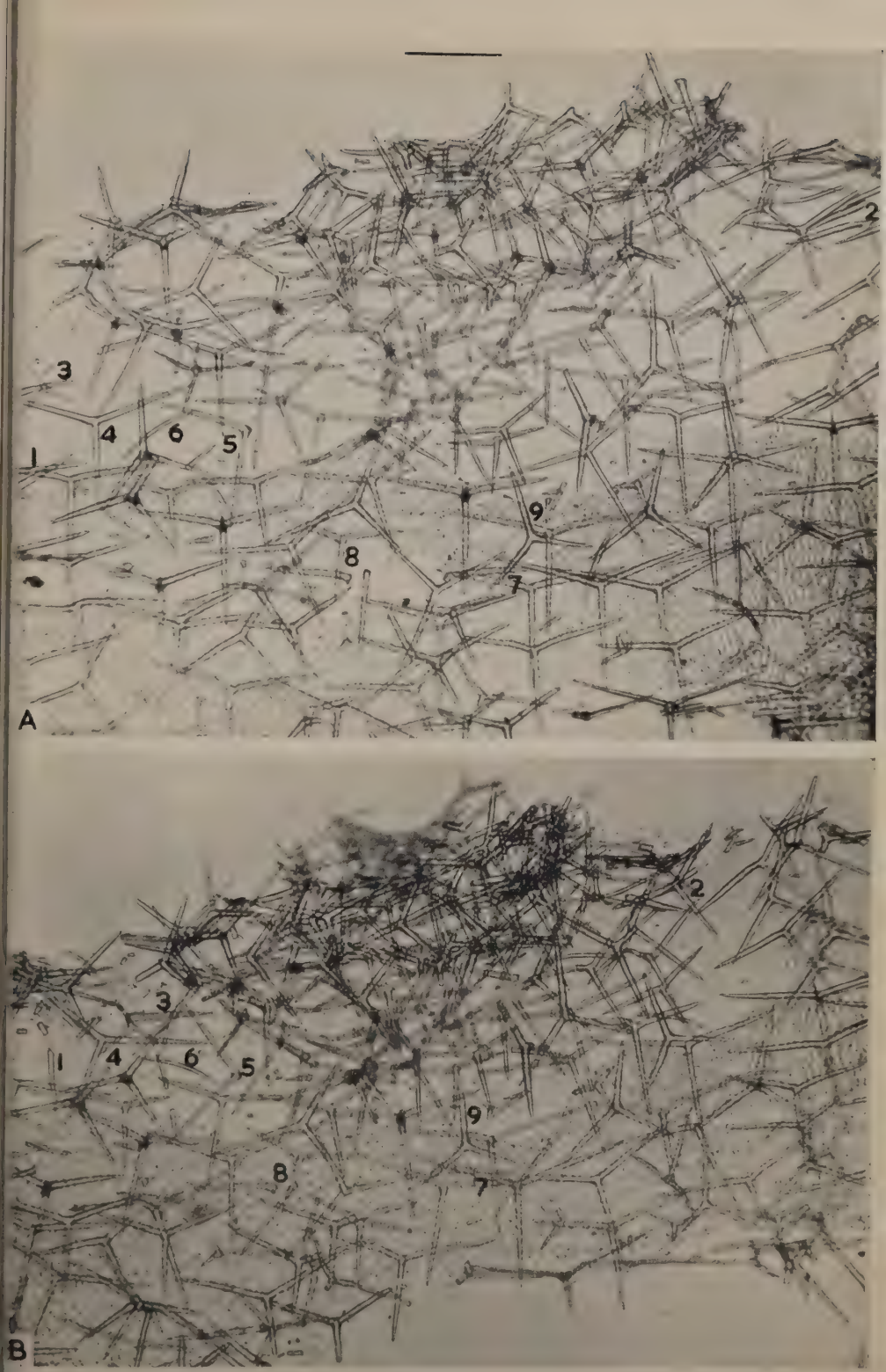

FIG. 3

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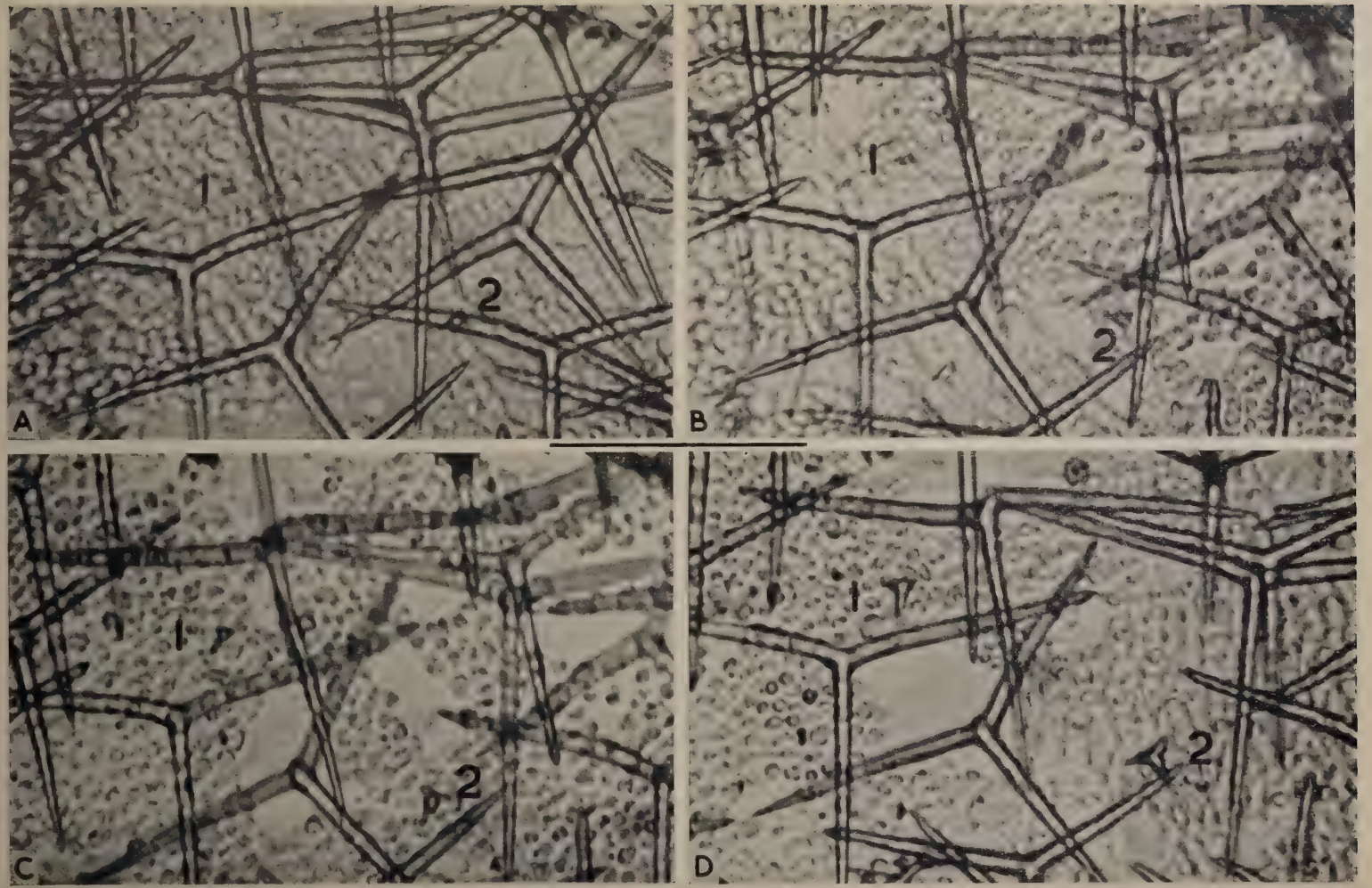
1
2
A
1
2
B
1
2
C
1
2
D

thelium. Spicule 3 in fig. 2 is an example. It has no associated calcoblasts and was probably attached to the upper epithelium by its rudimentary gastral ray. The absence of calcoblasts here was probably a result of the severe stretching. Eventually this spicule became rotated by the movement against it of spicule 5.

In fig. 4 it can be seen that the two small spicules rotated similarly without the direct intervention of other spicule rays. Further, their rotation was too great to be ascribed to a corresponding rotation of the epithelium to which they were attached, on the assumption that this was continuous. However, in the case of spicule 2 the rotation was accompanied by a displacement of the spicule relative to the large spicule rays in the vicinity, and hence to the mesogloeal matrix. The other small spicule (1) rotated similarly at first, but without obvious displacement with respect to the surrounding spicule arrangement, though even here a small displacement relative to the matrix cannot be excluded. Subsequently this small spicule rotated clockwise through nearly 90°, while spicule 2 retained the orientation shown in fig. 4, D. The area shown in fig. 4 actually lay behind and to the left of the area depicted in fig. 3, and at the end of the experiment spicule 1 had its basal ray pointing away from the contracting area of the latter figure, while spicule 2 had its basal ray pointing towards the gap which is partly visible at the bottom right corner of fig. 3, B.

Almost invariably the spicules which underwent considerable rotation were tiny, with a basal ray of about 10 μ or less in length. It was only in severely stretched regions (for example, fig. 2, C, D), or contracting regions (fig. 3), that large spicules rotated by more than a few degrees. Presumably the tiny spicules were more susceptible to rotation because their small size lessened the resistance encountered. Possibly the mesogloeal matrix was more fluid in their vicinity, while another factor perhaps would be the precocious development of the basal ray, for this enhances the bilateral symmetry of the spicule. It is not until the basal ray is about 15 μ long that the 3 rays become roughly equal in length (Jones, 1959). Before this stage a movement of the mesogloeal matrix past the spicule might cause rotation so that the axis of symmetry would coincide with the direction of movement, the calcoblasts moving into a disposition tending to minimize the resistance to the shear. At all events, the conditions affecting each small spicule on the stretching pieces would not be uniform in view of the irregularities in extensibility of the various parts of the piece, so that a consistent pattern of orientation of the small spicules would be exceptional. Much would depend on the size of each spicule and on local variations in displacement. It is not surprising, then, that small spicules should have rotated in different directions, even though they were fairly close together, nor is it surprising that some should have become oriented more or less at right angles to the applied tension, while others aligned themselves with it. Often there was no change in orientation at all (for example, sp. 9 in fig. 3).

FIG. 4 (plate). A–D, successive photographs showing the rotation of two small growing spicules. The photographs were taken at 1½, 3¾, 6, and 10 h respectively after the onset of stretching. Note the considerable displacement of the spicule rays in the vicinity of spicule 2. The line in the centre indicates 100 μ and the approximate direction of extension.

However, these inconsistencies are of relative unimportance when one is considering the mechanism of orientation in the normally developing oscular tube, for the orientation here in fact does not involve a rotation of the small spicule primordia from an initially random arrangement; the spicules grow in their proper orientation right from the first calcite rudiments to be laid down. It is the formative calcoblast clusters which adopt the appropriate arrangement to ensure the proper orientation of the spicule, so that the factors causing the spicule rotation referred to above are only of significance in so far as they do not exist to disturb the previously determined orientation of the spicules. It can be seen by comparing A and B in fig. 4 that the displacement of the small spicule 2 in a direction roughly parallel with its axis of symmetry has not resulted in much rotation. It can be concluded that if, in the normal oscular tube, the calcoblast clusters are oriented by a shear of the mesogloeal matrix in the direction of the oscular rim (Jones, 1954*a*), this shear would not disturb the orientation of the small spicules already in course of growth.

Orientation patterns of spicules in stretched pieces of the wall

As was indicated above, no consistent major change of orientation pattern was found with growing spicules on the photographed pieces. A pattern of reversed orientation was, however, obtained in 1949 on an unphotographed piece which had been mounted in essentially similar apparatus for 23 h (the maximum for the photographed material was 17 h). The basal end of this piece broke away from the clip on one side, so that the detached corner became drawn almost to the middle of the edge by the stretching process. With the continued extension the basal edge became more even, the corner projecting less and less, and examination of the subsequently fixed piece showed that this process was accompanied by shrinkage in the vicinity of the edge; the fully grown spicules were here crowded together. Further examination revealed that in the areas behind the basal edge the small spicules tended to be oriented with their basal rays directed away from the projecting corner; thus just behind this corner they were oriented in the opposite direction to the fully formed spicules, while along the basal edge on each side their basal rays tended to be roughly parallel with the edge. Near one of the sides, however, they were once again mostly oriented with their unpaired ray pointing away from the edge, again away from a site where crowding of spicules had occurred, though to a less extent than in the middle.

The occurrence of these areas of reversed orientation is in every way similar to the development of a zone of reversed orientation at the distal end of oscular tubes which have been mounted at their oscular ends on fine jets for at least 20 h (Jones, 1958). Here also the reversed pattern was associated with crowding of spicules at the edge, and it appeared after a similar period of time. The establishment of the reversed field at this time would cause the newly forming spicules to grow in the reversed orientation and might also bring about the rotation of existing tiny spicules. In the unphotographed piece referred to above, the largest reversed spicule had a basal ray 30 μ in length. Since the

measured growth rates of similar spicules increased to about 2·5 μ per h for rays exceeding 5 μ in length (Jones, 1959), it is probable that even this spicule began its development after the piece had been mounted. A 30 μ ray would take at least 12 h to grow, and if no rotation occurred, this would imply that the reversed field was established within 11 h of the piece first being mounted. However, a reversed pattern was never observed on any tube or piece mounted for 20 h or less; so it is probable that this spicule actually did rotate.

The crowding of spicules may have been caused either by a contraction of the epithelia at the edge, as in the example depicted in fig. 3, or by a shrinkage (absorption?) of the mesogloea in the crowded region. In the former case the small spicules attached to the upper epithelium would tend to be dragged towards the contracting region, whereas in the latter they might be carried in the opposite direction through the mesogloeal matrix. In the absence of a photographic record one cannot decide between these alternatives.

As described above, fully grown spicules experienced some rotation in regions of contraction, and fig. 3, B shows well the inclination of the spicules towards the contracting area. The rotation of the spicules here is simply due to the contraction occurring mainly at the edge itself; as the spicules were brought to the edge by the continued stretching, their oscular rays entered the contracting region and so the spicules became inclined.

One further type of reorientation is worth mentioning. When pieces of *L. complicata* are stretched, the slender monaxons become aligned in the direction of the applied tension, except for a few which remain at right angles to this. The reason is clear. The monaxons pierce the pinacoderm and are deeply embedded in the mesogloea, so that as stretching proceeds they will be swivelled about the place of insertion by the extension of the material in the vicinity of the embedded portion. Only those which lay in a plane exactly perpendicular to the applied tension would not become aligned with the direction of stretching. This suggests a mechanism for the orientation of monaxons. Over most of the oscular tube the slender monaxons tend to be perpendicular to the surface, but near the oscular end they tend to lie in axial planes of the tube and are inclined towards the oscular end (Jones, 1954*a*). According to Minchin (1908) one of the two formative cells migrates towards the gastral surface and makes contact with the bases of collar-cells there, while the other remains in or near the pinacoderm. The growing spicule primordium thus becomes suspended between the inner and outer epithelia, and it is clear from Minchin's fig. 42 that this primordium may be inclined to the pinacoderm before it has penetrated through this layer. It seems probable, therefore, that the inclination of the monaxons at the distal end of the tube is caused by a shift of the internal epithelium basalwards relative to the pinacoderm; it is certainly not due to the external water-currents set up by the outflow from the osculum, as might have been suggested.

A basally directed shift of the internal epithelium could arise as a result of the formation of new porocytes from the epithelium lining the oscular rim (endopinacoderm). In species of *Clathrina* the endopinacocytes show clear

stages of transformation into porocytes (Minchin, 1898). This indicates that, as the tube grows in length, the endopinacocytes progressively become displaced from the oscular edge. Indeed in *C. cerebrum* the process of transformation into porocytes begins on the outer surface of the tube, namely in the exopinacoderm. Presumably the differentiating cells here pass gradually forwards to the edge and then over this and back down the inner surface of the oscular rim (Minchin, 1898). In *Leucosolenia* porocytes also arise from the epithelium lining the oscular rim (Minchin, 1908) and I have been able to confirm, by photographically recording the growth of an oscular tube of *L. variabilis*, that the pores gradually become displaced from the oscular edge. Thus it is only necessary to assume that cell-division in the endopinacoderm occurs mainly at or close to the oscular edge (namely, distal to the zone of differentiation) while that in the exopinacoderm takes place more uniformly over the surface of the oscular rim, in order to explain the orientation of the slender monaxons by a proximally directed shift of the internal epithelium relative to the exopinacoderm. Further, the growth of the oscular tube is probably facilitated by the pressure of the mesogloeal matrix against the epithelium at the oscular edge, and since it is likely that the matrix is secreted beneath the choanocytes (presumably the main feeding elements in homocoelous sponges), the growth of the endopinacoderm at the oscular edge would entail a distally directed movement of the matrix past the internal epithelium, at least in the distal portions of the tube. Such a movement has been postulated to account for the orientation of the calcoblast sextets which lie in close contact with the internal epithelium, and so for the orientation of the triradiate spicules (Jones, 1954*a*, 1958). A more detailed consideration of the hypothesis of mesogloeal shear will be given elsewhere, but for the present it should be emphasized that even though the mesogloeal matrix tenaciously embeds the fully grown spicules, it can also undergo plastic deformation suggestive of the properties of a viscous fluid. It has also been demonstrated that spicules can be attached to the surface epithelia and can be displaced with respect to the mesogloeal matrix.

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Properties of the Wall of *Leucosolenia variabilis*

II. The Choanoderm and the Porocyte Epithelium

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With two plates (figs. 1 and 2)

Summary

Photographic records of extending pieces of the wall indicate that the choanocytes were but little affected by the stretching process. By adjusting their contacts mutually and by altering their positions on the wall, they avoided becoming greatly stretched in the direction of the applied tension.

Collar-cells cohere together and appear to be under tension in the expanded oscular tube. They also adhere to the inner surface of the wall. There was no evidence of attachment between spicules and choanocytes, and none for a transformation of the latter into pinacocytes, or conversely.

The pattern of pores changed considerably as the pieces extended, pores becoming obliterated and new ones opening up elsewhere. The pores never became slit-like, despite the considerable extension achieved by the pieces. Where pores persisted for a sufficient period, evidence was obtained to support the hypothesis that the porocytes are interconnected to form an epithelium on which the choanocytes can move about and to which, on its other surface, the founder calcoblasts of developing tri- and quadri-radiates are attached.

Introduction

In an earlier communication (Jones, 1957) a discussion was given on the nature of the tissue lining the spongocoel in species of *Leucosolenia*, and it was tentatively concluded that the porocytes were directly interconnected to form an epithelium on which the choanocytes could move and through which they could also pass. Further evidence in favour of this idea has been gained from the photographic records of extending pieces of the wall of *L. variabilis*, Haeckel, which were used for the measurement of the growth-rates of the spicules (Jones, 1959). This evidence will now be presented. The method used for stretching and photographing the pieces has already been described (Jones, 1959).

Observations

The choanoderm

It is well known that choanocytes vary in form (Duboscq and Tuzet, 1939), but they usually have cylindrical bodies projecting from the inner surface of the wall. These appeared in the photographs as rounded structures, separated from their neighbours by clear spaces, and as the wall was stretched they tended to remain roughly rounded in cross-section and did not separate much (fig. 1, D–F). The choanocytes in fact seemed to be but little affected by the

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stretching process. Their flagella remained beating, and though their bodies did spread somewhat in the central region of the wall, it was only in the lateral regions, where the wall became considerably extended, that the collar-cells spread out markedly over the surface. Even here they tended to remain roughly isometric in the plane of the wall.

A rough idea of the spread of the choanocytes was obtained by counting their numbers in squares of sides 25 μ long, taken more or less at random on areas between spicule rays. For the purpose the enlarged prints used for the measurement of the rays of growing spicules were employed. For one piece the average number per square of side 25 μ was 15·1 (8) at the start and fell to 9·7 (8) after 2 h and finally to 8·8 (31) after 11 h. The figures in brackets are the numbers of observations from which the averages were calculated. For another piece the number per square of side 25 μ declined more gradually from 16·3 (6) at 1½ h to 12·2 (14) at 10 h. The weights used to stretch the piece were then removed, whereupon the number rose to 13·5 (13) at 13 h and 13·9 (20) at 16 h. The pattern, however, remained much the same throughout: the choanocytes never became greatly extended in the direction of the applied tension.

The uniformity of the pattern of choanocytes, at any rate in the central parts of the extending wall, must have entailed readjustments in the connexions between the cells, as must also the opening and closing of the pores (see later). The collar-cells must also have been capable of adjusting their positions on the stretching wall, and this is confirmed by comparing the boundaries of the choanoderm in successive photomicrographs. As the wall was stretched its surface area increased, but the area covered by the choanoderm did not increase correspondingly, because the choanoderm narrowed more than the rest of the piece in the direction at right angles to the applied tension. This can be seen in the fig. 3 of my previous paper (Jones, 1961). The choanocytes were not greatly extended by the stretching of the wall, and by mutually adjusting their connexions one with another, and by moving over the wall, a retreat of the choanoderm occurred relative to the spicules and pores (fig. 2). The retreat was only relative; in absolute measure the choanoderm boundary approached the anterior edge of the piece as a result of the longitudinal shortening which

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FIG. 1 (plate). A, B, parts of the edge of a piece of the wall of *L. variabilis*. The piece was fixed and stained with picrocarmine immediately after excision. Note in A the closely aggregated choanocytes near the edge. Elsewhere they remain spread out. The act of cutting thus resulted in a recoil of the collar-cells at the edge. The recoil left an open porocyte (in A) and a calcoblast cluster (in B) exposed in the transparent wall.

C–F, part of an extending piece derived from the same oscular tube as the control above and photographed at 0, 3¾, 6, and 9¾ h after being mounted in the stretching apparatus. The choanoderm was damaged initially and the collar-cells recoiled to form a number of distinct islands of closely aggregated cells. Parts of 3 of these islands can be seen in C, with 5 open pores (arrowed) in the transparent wall in between. In D the collar-cells have spread across the gaps. The small spicule (encircled) rotated through more than 180° (C–F) as it grew. The rotation was accompanied by a considerable displacement from right to left between D and F.

The scale-line shown in A, B, and C represents 100 μ.

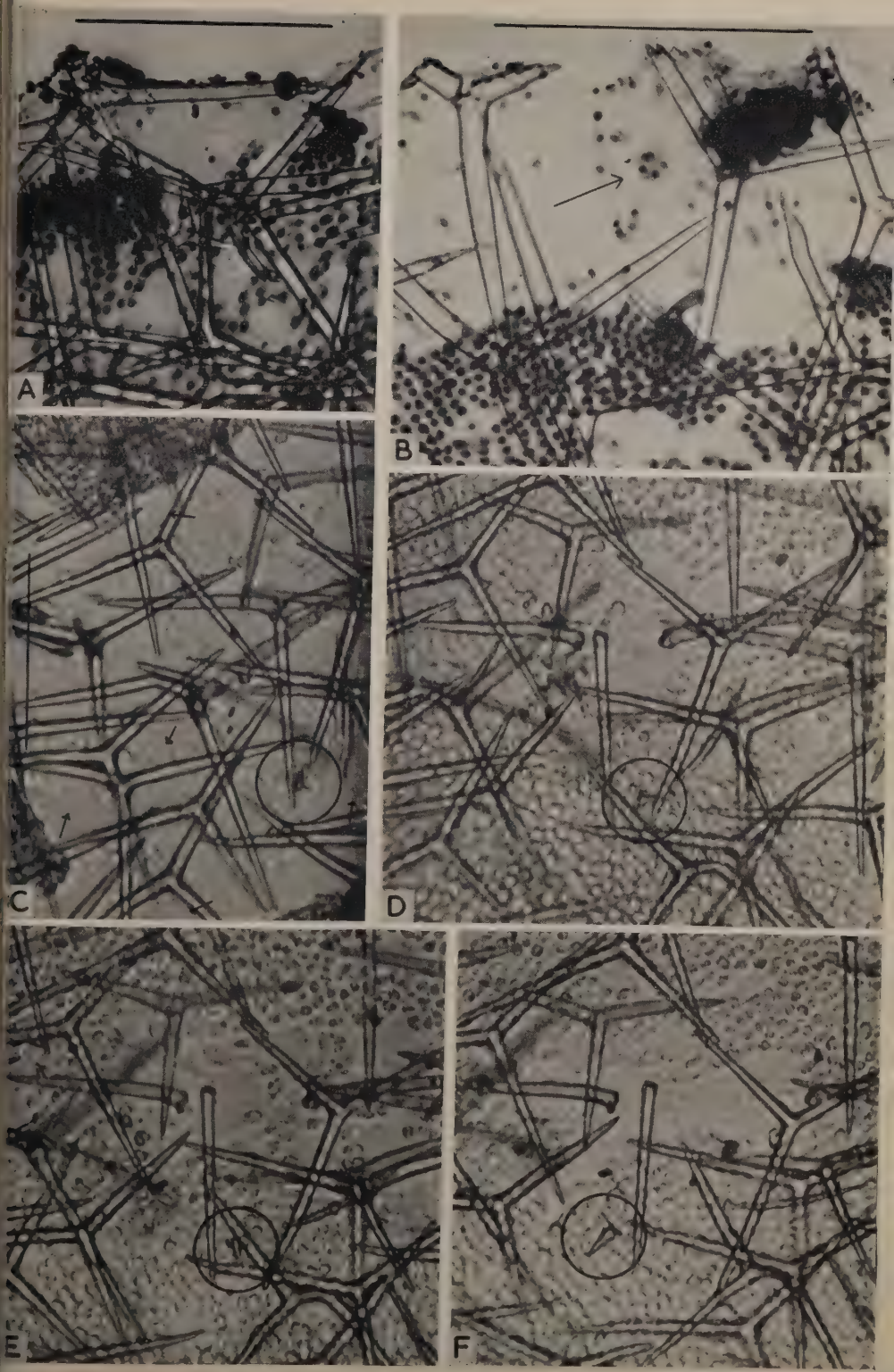

FIG. 1

W. CLIFFORD JONES

FIG. 2

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accompanied transverse extension. However, like the epithelia of the oscular rim (Jones, 1961), the choanoderm must be regarded as a tissue in which the cells, while tending to remain in contact with one another, are capable of readjusting their contacts. In fact the collar-cells behave rather like a layer of soap bubbles at an air-liquid interface. It should also be noted that even small growing spicules, which lie very close beneath the choanoderm (Minchin, 1908; Jones, 1954*b*), were left behind by the receding collar-cells (fig. 2, also fig. 3, Jones, 1961).

The ability of the choanocytes to move over the wall is also confirmed by the photographic record for another piece. The choanoderm on this was ruptured while the piece was being mounted in the stretching apparatus, and the damage resulted in a recoil of the collar-cells to form a number of separate islands of closely aggregated cells (fig. 1, C). One and a half hours later the choanoderm had become more or less continuous once more (fig. 1, D) and calculation shows that the islands had spread at a rate of at least 1 μ per hour. Presumably the flagellar cavity of the cells had driven the aggregates against the flattened wall and the cells had then spread until contact with other choanocytes, or with open porocytes, had been achieved. It should be noted that open pores and a growing spicule still remained in the transparent wall between the islands of collar-cells (fig. 1, C), and that the growing spicule subsequently rotated by more than 180° while undergoing displacement with respect to the other spicules in the vicinity, and hence to the mesogloeal matrix in which these were embedded.

The recoil of the ruptured choanoderm indicates that the choanocytes were under tension in the expanded oscular tube from which the piece was derived, and this is supported by examination of the control half of this tube. The tube in fact was bisected longitudinally, one half being fixed immediately as a control, and the other then stretched. On the control piece no islands of collar-cells can be seen, but at the cut edges some retraction and concentration of choanocytes has occurred (fig. 1, A, B). Over the rest of the piece the collar-cells remain uniformly spread out and attached to the wall. They must have been adhering to the substratum because their tension could not otherwise have been maintained, certainly not by the pegging action of the gastral rays of quadriradiate spicules, for on this piece very few quadriradiates were to be found. The act of cutting thus broke the adhesion between the collar-cells and their substratum in the vicinity of the cut, permitting recoil of the still

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FIG. 2 (plate). A, B, successive photomicrographs of part of a piece under tension, taken at 2 and 5 h respectively after the onset of stretching. Note the retreat of the choanoderm boundary relative to the pores (encircled) and spicules, including the small growing spicule (*X*) in the bottom left-hand corner. This spicule maintained roughly the same spatial relationship with the two pores to the right, but the two large triradiates in between became displaced to the left. The observations suggest that the growing spicule was in contact with a membrane which lay immediately beneath the choanocytes and which interconnected the gastral ends of the pores together. The triradiates, on the other hand, were embedded in the mesogloeal matrix. The scale-line between the two photomicrographs represents 100 μ.

cohering collar-cells to occur. As they recoiled under their pre-existing tension, open porocytes (fig. 1, A) and calcoblast clusters (fig. 1, B) were left behind in the transparent wall.

The adhesion of the collar-cells to their substratum is also shown by the maintenance of undulations in the choanoderm boundary as the wall is stretched (fig. 2), and by the spreading (and flattening presumably) of the collar-cells in greatly extended parts of the wall. The latter raises the possibility that the choanocytes may be able to transform themselves into cells of dermal type, but evidence for this was lacking in the photomicrographs and subsequently fixed pieces. There is no sign of a gradual conversion at the junction between the choanoderm and the internal epithelium of the original oscular rim (fig. 2). The retreat of the choanoderm boundary here is thus due to a genuine movement of the choanocytes, rather than to a transformation of collar-cells into pinacocytes. Minchin (1892) likewise could find no evidence for such a transformation in *L. clathrus*. It has long been known (Huxley, 1912) that clumps of collar-cells which have been teased from *Sycon* are unable to regenerate other types of cell.

The flattened collar-cells in greatly extended regions of the wall never betray any connexions with the tips of spicule rays. Only where these push up the choanoderm is there a correlated pattern of stretched collar-cells. Elsewhere, despite the movement of spicule rays with respect to the epithelia, the collar-cells show no signs of being pulled in any way.

Parts of the wall severed after a time between the jaws of the clips which were used to grip the piece, and the ruptured edges then became arched. When spicule rays projected from the edge, arching occurred from one projecting ray to the next, just as at the onset of the healing behaviour of isolated pieces of the wall (Jones, 1957). This arching involved a shrinkage or absorption of the mesogloeal matrix at the edge, for the extended choanocytes here became spindle-shaped, their long axes curving in the same way as the edge itself. Farther from the edge they remained large and spread out.

The pattern of pores

The positions of porocytes can easily be made out on the photographs when the pores are open (fig. 2, A), and in some cases one can even see the hole in the centre of the porocyte diaphragm (the membrane which juts across the pore canal, Minchin, 1898). In the other cases one simply sees a rounded gap in the choanoderm.

In the initial stages of stretching the pore gaps opened widely and became slightly elliptical, with the long axis of the ellipse along the direction of tension. They never became slit-shaped, however, even when the piece was greatly stretched; and this suggests that, like the choanocytes and epithelial cells of the oscular rim, the porocytes were constantly adjusting their connexions with neighbouring cells, in order to minimize the effect of over-extension in a particular direction. The arrangement of the pores changed considerably, many

pore gaps becoming obliterated and others appearing where none had existed before (fig. 1, E, F). One reason for the closure would be excessive strain caused by a displacement of the mesogloeal matrix: I have already demonstrated that the epithelia and mesogloea can move relatively to one another owing to irregularities in the properties of the 3 layers (Jones, 1961). Another reason would be the movement of spicule rays against the pore canal. Sometimes a ray could be seen projecting across a pore gap, and in such cases it is probable that the pore had closed and either the canal had been deflected by the ray, or the porocyte had severed its connexion with the pinacoderm. Such gaps were usually obliterated by choanocytes in the next photomicrograph of the area concerned.

It is possible that the porocytes can detach themselves from and re-attach themselves to the pinacoderm whenever the wall becomes excessively distorted by the stretching process, for Minchin (1900, fig. 42) has shown that the porocytes which arise from the epithelium lining the oscular rim of *Clathrina* first extend their bodies out to the pinacoderm, before becoming perforated by their pore canals. Minchin (1892*a*) also believed that porocytes could arise by the inward extension of cells belonging to the pinacoderm, and while this would account for the appearance of new pores on the stretching pieces, it seems more probable that porocytes already in contact with the choanoderm would be concerned; porocytes which had contracted would presumably reopen their pores sooner or later.

On the stretching pieces the number of pores per unit area was variable, as would be expected from the alterations in the pore pattern. In one piece the number remained at roughly 4 or 4·5 per square of side 100 μ for the first 4 h and then dropped as the arrangement became much less uniform. In healthy oscular tubes the number may be as high as 6 or 7 per square of side 100 μ. The alteration in pore pattern makes it difficult to follow the fate of particular porocytes for more than a few hours, but where this is possible there are clear indications that the porocytes were connected in some way with those spicules which can be assumed to have been attached to the upper epithelium (the pieces were mounted with their choanoderm uppermost). Such spicules would be those in the course of development, or those which had just completed development (Minchin, 1908; Jones, 1954*b*). In fig. 2, on the left, can be seen a small, growing spicule (*X*). In the interval between A and B the triradiate spicules in the immediate vicinity became displaced to the left with respect to this small spicule, but the two pores slightly farther to the right remained at more or less the same distance from it. They also retained the same spatial relationship with the quadriradiate spicule farther to the right, which would also tend to be anchored to the upper epithelium by its gastral ray. These observations can be explained by assuming that the triradiates were embedded in the mesogloeal matrix, and that here this became displaced to the left with respect to the upper epithelium, to which the porocytes and other spicules mentioned above were attached. The connexions between the pores and the associated spicules were presumably not mediated by the collar-cells, since

the choanoderm boundary receded relatively to the pores and the growing spicule (fig. 2). It is not likely that the mesogloeal matrix maintained the connexions, since the displacement of the triradiates in the vicinity would seem to preclude the possibility that the matrix was sufficiently firm. The evidence thus suggests that there is a membrane interconnecting the gastral ends of the pores, and that the collar-cells rest on the upper surface of this membrane, while the developing spicules are in contact with its lower surface.

Discussion

The 'porocyte epithelium'

It has been shown above that displacement of the collar-cells does not interfere with the development of small spicules lying close beneath, and that the collar-cells do not appear to be attached to calcoblast clusters or to the rays of fully grown spicules. Moreover, an example has been given in which a small spicule maintained the same spatial relationships with pores in its vicinity while both the choanoderm and the mesogloeal matrix (as indicated by embedded triradiates) were being displaced in different directions with respect to them. The evidence thus suggests that there is a membrane between the mesogloea and the bases of the collar-cells—a membrane to which both the porocytes and the developing spicules are attached, and on which the collar-cells can move about. However, since the porocytes are derived from the endopinacoderm which lines the oscular rim (Minchin's 'porocytic epithelium' (1898)), developing their pores only when overgrown by the choanoderm (Minchin, 1898; Jones, 1952), one may suppose that these cells retain their interconnexions after their pores have opened, and that the membrane referred to above is simply a continuation of the epithelium lining the rim. Further evidence for this has already been presented (Jones, 1957), and at that time it was considered that the 'porocyte epithelium' beneath the collar-cells must be perforated, since collar-cells can pass through into the mesogloea (Gatenby, 1920; Duboscq and Tuzet, 1936, 1939). However, this is not necessarily so, for leucocytes are known to be capable of penetrating through the endothelial cells lining the blood-capillaries of mammals, and the endopinacocytes of sponges seem in many ways to be similar to these endothelial cells.

The existence of a 'porocyte epithelium' (a convenient term would be 'poroderm') beneath the collar-cells is difficult to demonstrate by ordinary histological techniques, but Eberl-Rothe (1957) has discovered a silver-staining 'basal membrane' beneath the choanocytes of a sponge labelled *Ascetta* (*Leucosolenia*). In another example of the genus, from a different locality, the basal membrane was not visible but instead she found that the choanocytes sent processes down into the mesogloea, while at their bases lay semicircular cells, which she believed to be potential choanocytes. Such differences in structure between two closely related species probably reflects a difference in the physiological state at the time of fixation, and this is supported by the variety of the results of Eberl-Rothe's investigations of other species: thus in

Sycandra capellosa she found both a limiting membrane and flattened cells at the bases of the choanocytes; in *Grantessa* she found a membrane beneath the small basal cells; while in *Grantia* the membrane appeared to be like that of *Ascetta*, except that the basal cells were also present, though widely separated. One can explain these differences largely in terms of different states of health and contraction of the material at fixation. Basal filaments occur on the choanocytes in contracted radial tubes of *Sycon* (Duboscq and Tuzet, 1939) and were previously described by von Lendenfeld (1892) for several calcareous sponges, including *A.* (= *Leucosolenia*) *primordialis*. According to Minchin (1892*a*), however, they are not a feature of the normal collar-cells of *L. clathrus*; he saw these processes being emitted by the collar-cells in life, but considered them to be a sign of imminent cessation of activity and death. Rio-Hortega and Ferrer (1917) also saw processes exceptionally on some collar-cells of *L. falcata*, and I have deduced that they must be present when tubes of *Clathrina* are undergoing the extreme contraction described by Minchin (1900) (Jones, 1957). There can be little doubt that when such processes occur in sections, they would obscure the thin basal membrane.

As regards the basal cells, Duboscq and Tuzet (1939) have already discovered the existence of a layer of flattened collencytes beneath the collar-cells of *Sycon*, and there seems no reason why in *Leucosolenia* the basal cells should not have been contracted porocytes, of which no mention was made by Eberl-Rothe. If so, the spacing apart of these cells would depend on the degree of contraction of the material, as would also the thickness (and hence distinctness) of the 'membrane' connecting these cells. Eberl-Rothe's results can thus be regarded as supporting the view that the porocytes of *Leucosolenia* are interconnected beneath the bases of the collar-cells. This could be confirmed with the aid of the electron microscope.

The mechanism of spicule orientation suggested in my previous papers (Jones, 1954*a*, 1958, 1961) requires that the calcoblast sextets should be in contact with an epithelium which becomes progressively displaced basalwards with respect to the mesogloeal matrix as the oscular tube grows in length. The choanoderm, consisting as it does of apparently mobile choanocytes, and presumably growing lengthwise in much the same way as the mesogloeal matrix, would not have the properties demanded by the hypothesis. The 'poroderm' referred to above, however, would possess the requisite stability and growth characteristics, since porocytes are formed at the oscular end of the tube while the mesogloeal matrix is probably being secreted in the region of the choanoderm (the main feeding area). Proof of the existence of the 'poroderm' beneath the bases of the collar-cells is thus vital to the hypothesis as it stands at present.

The mobility of the choanocytes

The ability of the choanoderm to move about over the stretching wall of *Leucosolenia* is not surprising, since it has been known for many years that isolated collar-cells can move in amoeboid fashion, even while still beating their flagella (Carter, 1869). This is not so for all sponges, however. In fresh-water

species the collar-cells move weakly or not at all when isolated (Brøndsted, 1936), while in the living sponge they have not been observed to put forth pseudopodia (Wintermann, 1951). In fact they strongly cohere to form the unilayered walls of the flagellated chambers (Wintermann, 1951; Kilian, 1952). These are attached to the walls of the excurrent canals at their apopyles and project freely into the incurrent canals. No cells or membrane lie beneath the collar-cells (Wintermann, 1951; Kilian, 1952; Rasmont, 1959), though their bases may be in contact with processes of collencytes. The arrangement thus seems to be quite distinct from that of *Leucosolenia*. However, in respect of the pores through which water enters the sponge there would be a striking similarity to *Leucosolenia* if the existence of the 'poroderm' in this genus were confirmed, for in fresh-water sponges the pores are situated intracellularly within endopinacocytes, and intercellularly between exopinacocytes, of the dermal membrane, which consists of an outer exopinacoderm, a middle connective-tissue layer, and an inner endopinacoderm (Ankel and Wintermann–Kilian, 1952), just as would also the dermal part of the wall of *Leucosolenia*.

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a, aster; *ab*, acroblast; *af*, axial filament; *as*, acrosome; *b*, basal granule (blepharoplast); *bb*, brush border; *bm*, basement membrane; *c*, centriole; *ch*, chromosome; *cl*, cilium; *cm*, cell membrane; *ctm*, centromere (kinetochore); *cts*, centrosphere (idiozome); *er*, endoplasmic reticulum (ergastoplasm); *f*, flagellum; *g*, *γ*-cytomembrane ('Golgi' membrane); *l*, lipid droplet; *m*, mitochondrion; *n*, nucleus; *nl*, nucleolus; *nm*, nuclear membrane; *s*, spindle; *v*, vacuole.

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